Determination of Amino Acids in Industrial Effluents Contaminated Soil

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Summary: 38 samples of soil for 19 locations partially irrigated on the effluents of sugar mill and oil andghee mill, bottom sediments of evaporation ponds of sugar and fertilizer industries were collected and analyzed for amino acids after acid digestion by gas chromatography using pre column derivatization with trifluroacetyleacetone and ethyl chloroformate. The results obtained were compared with the soil samples irrigated with fresh water. The soil samples were also analyzed for pH, total nitrogen contents and organic carbon. Nine essential {leucine (Leu), threonine (Thr), lysine (Lys), L-phenylalanine (Phe), tryptophan (Trp), histadine (His), L-valine (Val), methionine (Met) and isoleucine Ile) and ten non-essential (alanine (Ala), cysteine (Cys), asparagine (Asn), glutamic acid (Glu), serine (Ser), glycine (Gly), proline (Pro), Glutamine (Gln), aspartic acid (Asp), tyrosine (Tyr)} amino acids were analyzed 13..15 amino acids were identified and determined quantitatively from soil samples. Amino acids Met, Asn, Gln and Trp were observed absent from all the samples. The variation in the amino acids contents in soil with the industrial effluents added and total nitrogen and organic carbon is discussed.

Key words**:** Amino acids, Effluents, Irrigated, Land, Environmental, Pollution**.**

Introduction

The determinations of amino acids in soils have remained of analytical interest to agricultural chemist for several years. The sources of amino acids nitrogen results from plant debris, micro flora and their decomposed products and metabolites. Organic nitrogen is an important pool of soil N and is a source of plant available minerals N [1]. The identification of soil organic N hydrolysates indicates amino acids 33 to 42% of total N contents [2]. Schulten and Schnitzer have reported little variability in soil amino acids composition and thus suggested the usefulness of soil amino acids concentration as an indicator of any biological property [3, 4] observed that amino acids and amino sugars were indicator of soil N responsiveness by agricultural crops. The free amino acids could be determined in water [5], but the quantitative determination of total amino acids N requires rigorous acid hydrolysis to break the amino acids bonds present in polymeric amino acids compounds such as peptides and proteins [6]. Untreated sewage water and industrial effluents are allowed to drain in agricultural lands and may affect the composition of the soil. A variation in analysis of the soil including organic carbon, total nitrogen and total amino acids contents in the affected soil as compared to unaffected soil may reflect the extent of the contamination. Little information is available on the effect of industrial effluents on the amino acids composition of soil organic matter. Such information may be needed because of continual emphasis on sustainable agriculture and soil environmental quality in evaluating the effects of different soil management systems on the N dynamics of soil.

A number of methods have been applied for the determination of amino acids in soil samples. The analytical procedures commonly used are based on liquid chromatography (LC) [7, 8], gas chromatography (GC) [9, 10], ion exchange chromatography (IEC) [11] and capillary electrophoresis (CE) [11-13], Pre and post column derivatization could be used for LC for determination of amino acids but pre column derivatization method is considered more promising for amino acids analysis by LC [14]. The commonly used derivatizing reagents include ortho-phthalaldehyde [15], phenyl isothiocyanate [16], 2-(9-fluorenyl) ethyl chloroformate [17], 5-dimethylamino-1-naphthalene sulphonyl chloride (dansyl chloride) [18] and 6-aminoquinolyl-N-hydoxysuccinimidyl carbamate [19]. A number of difficulties associated with LC procedure have been described [8]. GC has high resolution power for the separation of organic compounds with ease of operation with lower running cost. The analyses times by GC are also generally short. The amino acids are non-volatile and require pre column derivatization. Different silyl derivatives [20, 21], ethyl chloroformate [22, 23] and their related compounds [24-26] are used as reagents for GC determination of amino acids. Recently

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trifluoroacetylacetone (FAA) and ethyl chloroformate (ECF) have been used for two stage derivatization for GC determination of amino acids [10]. The present work examines the amino acids composition of soil, organic matter from 38 soil samples under the influence of the effluents of sugar, oil and ghee and fertilizer industries and compares the results with unaffected soil. The potential use of the information could be valuable in characterization of soil health on quality (Table-1).

Results and Discussion

Soil Analysis for Amino Acids

The calibration curves were recorded for simultaneous determination of amino acids for each analysis by recording average peak height/peak area (n=4) against concentration within 2-12 µg/ml, followed by the analysis of the soil samples for amino acids. The values of coefficient of determination (R^2), limit of determination (LOD) and limit of quantification (LOQ) for all analyzed amino acids were ranged between 0.9921 – 0.9993, 0.1 – 0.3 µg/ml and 0.3 – 0.9 µg/ml respectively (Table-2).

A typical procedure for the determination of total amino acids in N-containing organic compounds was based on acid hydrolysis of the soil samples with 6N HCl in closed screw capped vial at 110^0C for 24h [27, 28]. The hydrolysates after necessary separation and derivatization were analyzed by GC. 19 amino acids were examined (Fig. 1), but 13-15 amino acids were identified and determined quantitatively (Fig. 2). The amino acids Met, Asn, Gln and Trp were observed below the quantification for all the samples analyzed. Met and Trp may not have survived hot acid hydrolysis and could not be detected [29, 30]. A soil sample (12A) after acid hydrolysis and extraction of amino acids in aqueous phase (Fig. 3) was spiked with amino acids standard at the final concentration of 5 µg/ml. GC analysis was carried out after chemical derivatization. An increase in the response corresponding to the standard added was observed, without change in peak shape of each amino acid (Fig. 4). The results indicate that the soil matrix did not interfere the determination of amino acid (Fig. 5) with the calculated recovery of amino acids within the range 92-98% with RSD within 1.6% (n=3) (Table-3).

Table-1: Mean values of physicochemical features of Soil, Sediments of Evaporation Ponds and Reference Soil Samples irrigated with industrial effluents and fresh water from Ghotki and Rahimyar Khan Districts, Pakistan

	pH	TN mg Kg-1	Org.Caron mg Kg-1 (% w/w)	C/N Ratio	Characteristics of Soil
JDW S. Mill Hor. A (n=5)	8.2	702	1040	1.4	Clay Loam
JDW S. Mill Hor. B (n=5)	8.3	586	984	1.8	Clay Loam
Ahmad O andGhee Hor. A (n=3)	8.3	770	1160	1.5	Clay Soil
Ahmad O andGhe.Hor. B (n=3)	8.3	746.6	1120	1.5	Clay Soil
G and G.M.S. Mill Hor. A (n=4)	8.2	1000	1470	1.4	Clay Loam
G and G.M.S. Mill Hor. B (n=4)	8.3	770	1160	1.5	Clay+ silty Loam
United S. M. SDK Hor. A (n=2)	8.2	870	1260	1.4	Clay+ silty Loam
United S. M. SDK Hor. B (n=2)	8.1	1000	1500	1.5	Clay Soil
FFC Machhi Goth Hor. AB (n=2)	7.8	800	1200	1.5	Clay Soil
Ref. SDK. Hor..A (n=2)	8.1	430	720	1.6	Clay Loam
Ref. SDK. Hor. B (n=2)	8.0	370	505	1.3	Clay Loam
Ref. Ghotki H or. A (n=2)	8.0	650	1095	1.6	Clay +Silty Loam
Ref. Ghotki H or. B (n=2)	7.9	495	915	1.8	Clay +Silty Loam

n = number of samples analyzed
Hor. = Horizon
A = at the depth of 0…15 cm
B = at the depth of 15…30 cm

Table-2: Analytical parameters for the amino acids analysis using FAA and ECF as derivatizing reagent.

Amino acid	LOQ(µg/ml)	LOD(µg/ml)	Calibration range (µg/ml)	Coefficient of determination R^2	Linear Regression equation
Gly	0.3	0.1	2-12	0.9966	y=3.4143x+0.9333
Ala	0.3	0.1	2-12	0.9977	y=3.3429x-1.4520
Val	0.3	0.1	2-12	0.9921	y=3.3857x+0.4667
Leu	0.3	0.1	2-12	0.9947	y=3.7643x+2.0667
Ile	0.3	0.1	2-12	0.9964	y=3.907x+1.4321
His	0.6	0.2	2-12	0.9976	y=3.2071x-0.3667
Ser	0.6	0.2	2-12	0.9986	y=2.0643x-0.7667
Thr	0.6	0.2	2-12	0.9968	y=2.7286x-2.2667
Cys	0.6	0.2	2-12	0.9924	y=3.7743x+2.3467
Met	0.6	0.2	2-12	0.9963	y=3.5286x+2.1333
Asp	0.9	0.3	2-12	0.9984	y=1.6532x-0.812
Asn	0.6	0.2	2-12	0.9963	y=1.7214x-0.533
Pro	0.6	0.2	2-12	0.9916	y=3.450x-1.0667
Glu	0.9	0.3	2-12	0.9993	y=3.0714x-1.6667
Gln	0.6	0.2	2-12	0.9949	y=3.6814x+1.7467
Lys	0.6	0.2	2-12	0.9928	y=3.5714x+1.3333
Tyr	0.6	0.2	2-12	0.9975	y=3.3857x-1.5333
Trp	0.6	0.2	2-12	0.9954	y=3.5533x+0.5667
Phe	0.6	0.2	2-12	0.9928	y=3.450x+0.2667

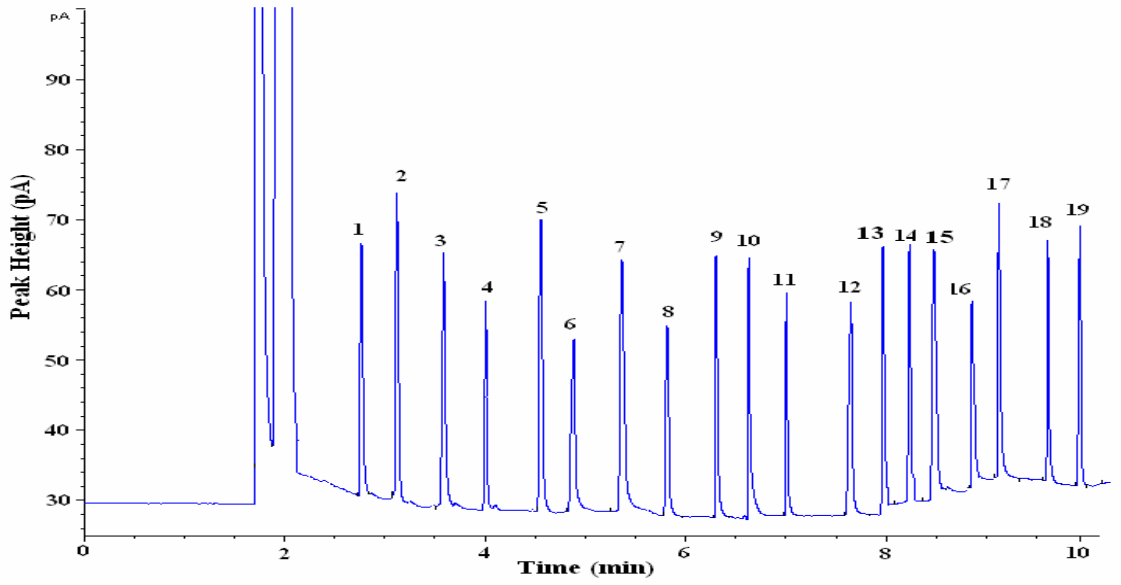

Fig. 1: Separation of amino acids using FAA and ECF as derivatizing reagents.
1. Ala, 2. Gly, 3. Val, 4. Leu, 5. Ile, 6. Pro, 7. Thr, 8. Ser, 9. Asn, 10. Asp, 11. Glu, 12. Gln, 13. Cys,14. Met, 15. Phe, 16. Lys, 17. His, 18. Tyr, 19. Trp. GC conditions: Column HP-5 (30 m × 0.32 mm id) with film thickness of 0.25 µm at column temperature 100^0 C for 2 min with ramping of 20 ^{0}C min^{-1} up to 250 ^{0}C with nitrogen flow rate of 3 mL min^{-1} with split ratio 10:1. The injector and detector temperatures were 270^0 C and 280^0 C respectively.

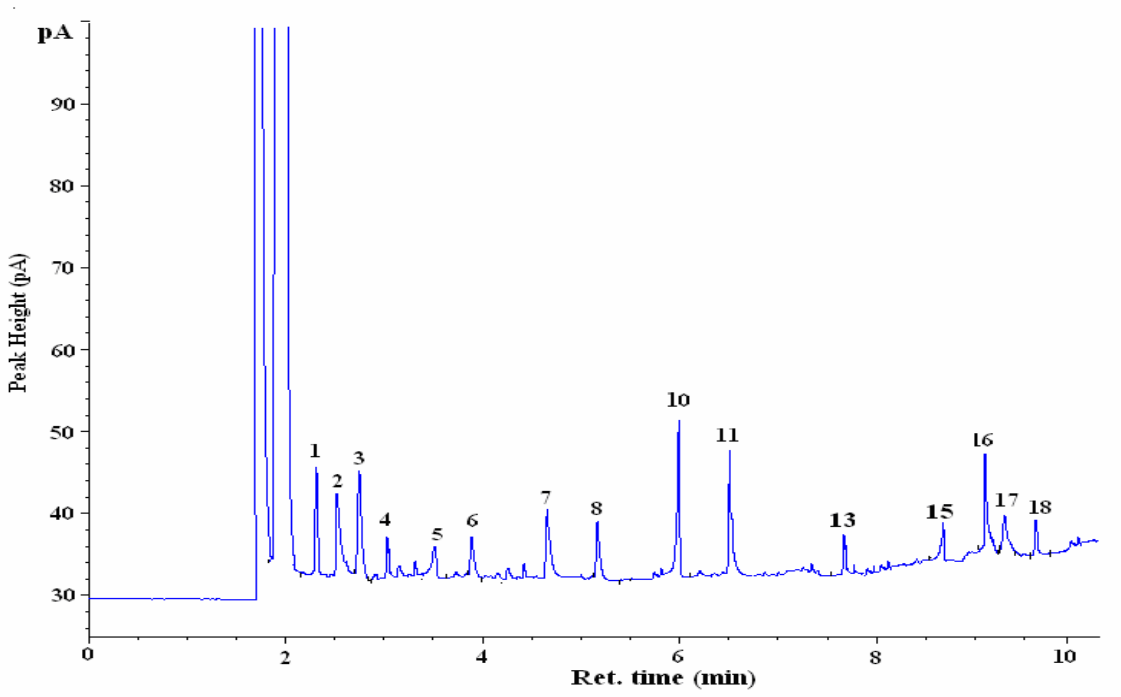

Fig. 2: GC response for acid hydrolyzed amino acids in soil samples collected from agricultural land receiving JDW sugar mill effluent
1. Ala, 2. Gly, 3. Val, 4. Leu, 5. Ile, 6. Pro, 7. Thr, 8. Ser,10. Asp, 11. Glu,13. Cys,15. Phe, 16. Lys,17. His,18. Tyr. GC conditions as (Fig. 1).

Table-3: Ghotki effluent irrigated Soil Sample of along with spiked value and percentage recovery.

Ghotki Sugar Mill			
S. No	**Soil Sediment**	**Soil Sediment spiked**	**Recovery % age**
Gly	**470**	**468**	**98%**
Ala	**750**	**728**	**97**
Val	**710**	**690**	**97**
Leu	**170**	**163**	**96**
Ser	**210**	**196**	**93**
Thr	**260**	**245**	**94**
Ile	**100**	**95**	**95**
Cys	**60**	**57**	**95**
Met	**-**	**4.5**	**92**
Asp	**1120**	**1065**	**95**
Asn	**-**	**6.5**	**92**
Glu	**960**	**890**	**93**
Gln	**-**	**6.3**	**94**
Pro	**110**	**103**	**94**
Lys	**450**	**438**	**97**
His	**70**	**65**	**93**
Phe	**90**	**85**	**94**
Tyr	**50**	**46**	**92**
Trp	**-**	**4.9**	**95**

∗ = Spiked Sample

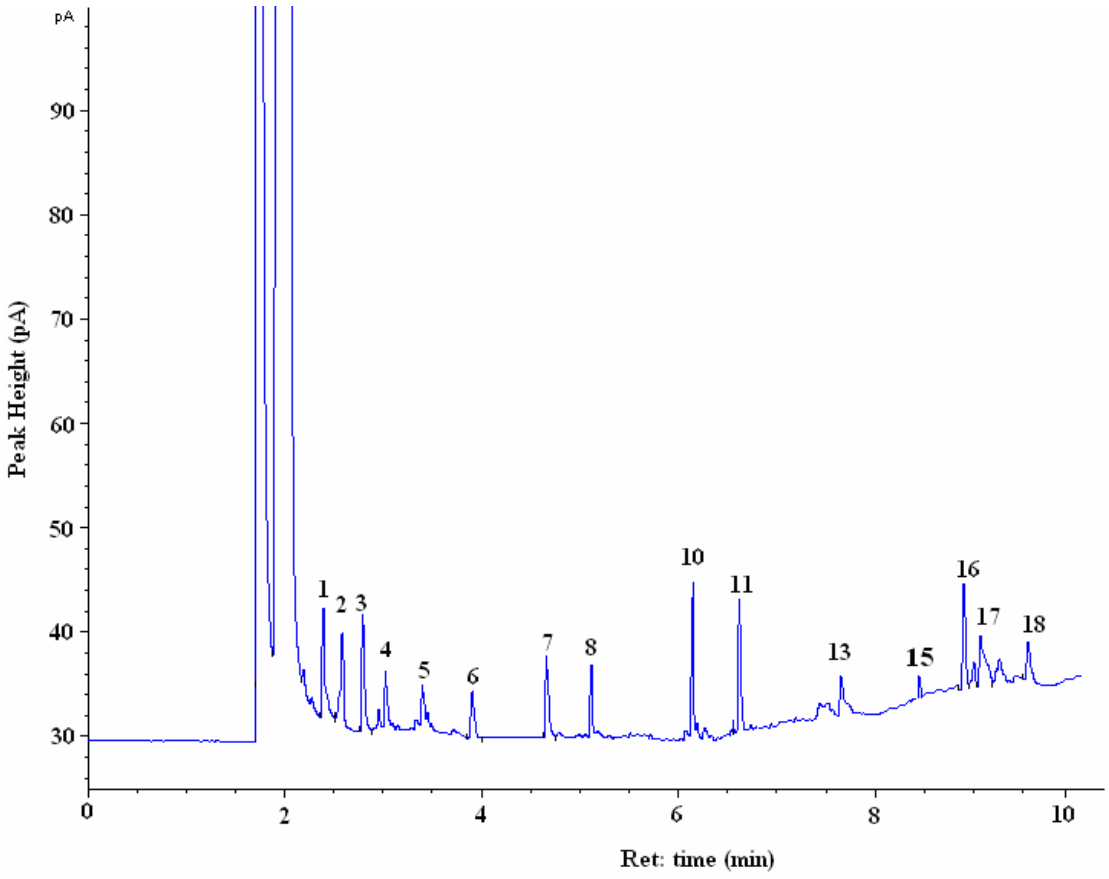

Fig. 3: GC response for acid hydrolyzed amino acids in (12 A) sample collected from effluent storing evaporation pond of Ghotki sugar mill.
1. Ala, 2. Gly, 3. Val, 4. Leu, 5. Ile, 6. Pro, 7. Thr, 8. Ser,10. Asp, 11. Glu,13. Cys,15. Phe, 16. Lys,17. His,18. Tyr. GC conditions as (fig 1).

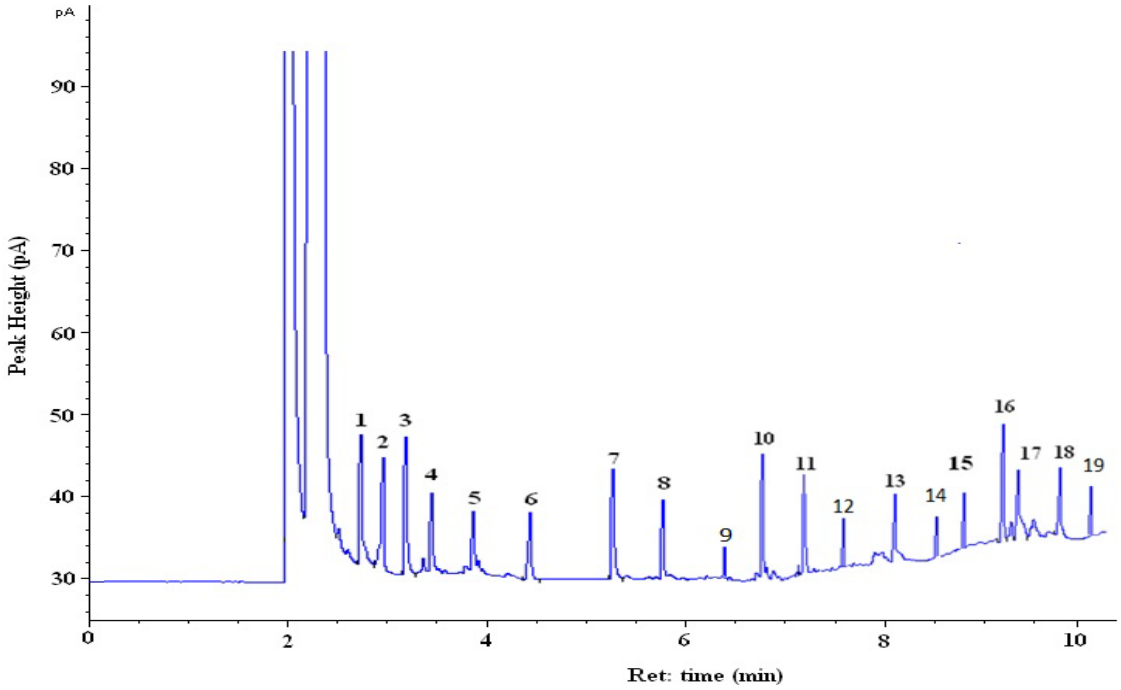

Fig. 4: GC response for spiked acid hydrolyzed amino acids in soil sediment samples collected from effluent storing evaporation pond of Ghotki sugar mill.
1. Ala, 2. Gly, 3. Val, 4. Leu, 5. Ile, 6. Pro, 7. Thr, 8. Ser, 9. Asn, 10. Asp, 11. Glu, 12. Gln, 13. Cys,14. Met, 15. Phe, 16. Lys, 17. His, 18. Tyr, 19. Trp. GC conditions as (fig 1)

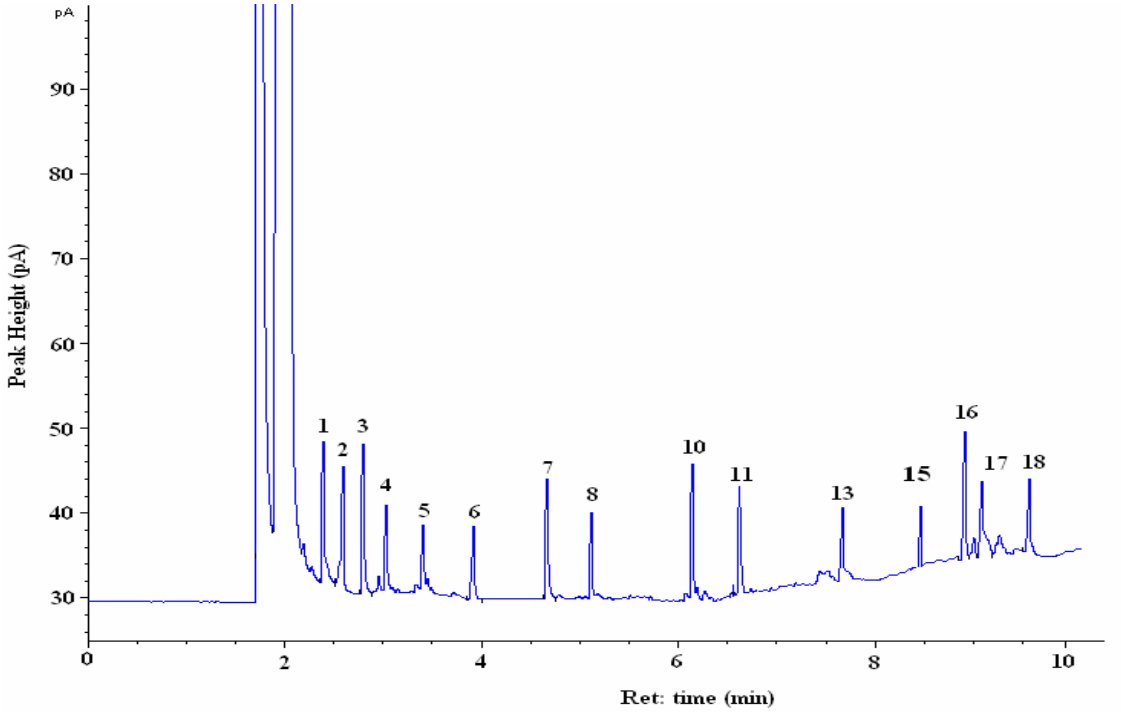

Fig. 5: GC response for acid hydrolyzed amino acids in soil sample collected from fresh water irrigated land (SDK reference soil sample (Sona Mahar))
1. Ala, 2. Gly, 3. Val, 4. Leu, 5. Ile, 6. Pro, 7. Thr, 8. Ser, 10. Asp, 11. Glu, 13. Cys,15. Phe, 16. Lys,17. His,18. Tyr. GC conditions as (fig 1).

The soil samples were collected from two levels A (0 - 15 cm) and B (15 - 30 cm) and composite sample from each was separately analyzed for amino acids concentration after acid hydrolysis. It was observed that samples at B levels contained mostly lower concentration of amino acids than A, may be due to consumption of organic nitrogenous compounds by microbes at deeper soil.

The average results of amino acids with confidence interval at 95% level for the soil samples receiving industrial effluents from Jamaluddin Wali (JDW) sugar mill, Ahmad oil and ghee mill, GMM. Sugar mill,United sugar mill, Fauji Fertilizer Company (FFC) and reference soil samples receiving fresh water for cultivation at level A and B are summarized in the (Table-4a, b). The soil receiving industrial effluent and reference soil collected from Tahsil Ghotki indicated highest concentration of the amino acids Asp > Glu > Ala > Val at level-A, but the reference samples collected from Taluka Sadiqabad indicated slightly different pattern with Asp > Val > Glu > Ala at A level. The observed relative concentration of amino acids in the soil receiving the effluents of JDW sugar mill at A level were with following decreasing order: Asp > Glu > Ala > Val > Gly > Lys > Thr > Leu > Ser > Pro > Ile > Phe > His > Cys. Similarly the soil receiving effluents of edible oil mill (oil and ghee) indicated the amino acids in following decreasing order:Asp > Glu > Ala > Val > Lys > Thr > Leu > Ser > Pro > His > Tyr > Ile > Cys > Phe. The relative amounts of amino acids was somewhat differed from reference samples from the same area using fresh water with following order:Asp > Val > Val > Glu > Ala > Gly > Thr > Lys > Leu > Pro > His > Ser > Cys > Tyr > Ile.

The concentration of amino acids detected were observed in the range for the soil receiving JDW Sugar mill effluent within 66-1074 mg Kg^{-1} and oil and ghee effluents within 60-1006 mg Kg^{-1} as compared to reference samples at the same level (A) within 35-690 mg Kg^{-1}. The average results of analysis of soil receiving effluents of JDW sugar mill and oil and ghee effluents were compared with the average results of reference soil samples from Sadiqabad (SDK) for amino acids.

The test for one way analysis of variance (ANOVA) was examined for the composition of amino acids contents in effluent irrigated soil (n=5) and reference soil samples (n=2) as reported [31]. The values of F-test were calculated at 95% confidence level and indicted a significant difference at the degree of freedom was indicated (Table-5a, b) for the amino acids (Ala, Val, Ile, Cys, Asp, Glu and Tyr) in soil receiving the industrial effluents and reference soil samples.

The pH of the soil under study based on water extracted at soil-water ratio (1:3 w/v) was slightly alkaline. The pH of soil receiving industrial effluents varied within 7.8 - 8.9. The highest pH was observed from the soil receiving industrial effluents of JDW sugar mill, Sadiqabad and lowest from the evaporation ponds of Fauji Fertilizer Company (FFC). pH of reference soil samples were observed within narrow range of 7.9 - 8.2 for both SDK and Ghotki Talukas (Table-1).

Table-4: (a) Mean values of concentrations of Acid hydrolysable Amino Acids in (mg/Kg) of Soil Horizon A and B irrigated with JDW sugar industry, Ahmad Oil and Ghee industries and G and G. M.M. sugar mill effluents. The values in Parenthesis indicate Confidence limit at 95% level.

	JDW Horizon A	JDW Horizon B	Ahmad oil and ghe Horizon A	Ahmad oil and gh, Horizon B	G and G.M.M.S. Mill Horizon A	G and G.M.M.S. Mill Horizon B
	Mean	Mean	Mean	Mean	Mean	Mean
Gly	472 (41)	404 (11)	473 (187)	450 (114)	465 (66)	410 (55)
Ala	732 (88)	736 (64)	700 (155)	636 (38)	797 (65)	780 (34)
Val	732 (69)	638 (48)	686 (38)	633 (100)	720 (59)	480 (265)
Leu	200 (12)	120 (29)	203 (28)	130 (65)	177 (8)	142 (27)
Ser	198 (27)	110 (41)	186 (57)	83 (51)	192 (20)	107 (49)
Thr	332 (52.4)	294 (59)	293 (38)	230 (41)	317 (96)	277 (47)
Ile	86 (14)	66 (16)	76 (14)	66 (28)	85 (20)	70 (26)
Cys	66 (7)	50 (02)	76 (38)	60 (0)	67 (8)	60 (5)
Met	BDL	BDL	BDL	BDL	BDL	BDL
Asp	1074 (54)	970 (50)	1006 (117)	853 (87)	1077 (45)	925 (42)
Asn	BDL	BDL	BDL	BDL	BDL	BDL
Glu	870 (51)	712 (12)	943 (125)	816 (274)	950 (93)	762 (129)
Gln	BDL	BDL	BDL	BDL	BDL	BDL
Pro	126 (18)	116 (10)	130 (43)	110 (25)	115 (30)	97 (15)
Lys	426 (85)	292 (14)	466 (23)	325 (186)	417 (91)	290 (132)
His	74 (7)	66 (104)	93 (14)	70 (24)	82 (33)	80 (13)
Phe	82 (37)	70 (27)	66 (28)	90 (0)	77 (24)	80 (2)
Tyr	72 (10)	64 (6)	83 (38)	75 (12)	82 (40)	66 (19.9)
Trp	BDL	BDL	BDL	BDL	BDL	BDL

Table-4: (b) Mean values of concentrations of Acid hydrolysable Amino Acids in (mg/Kg) of Soil Horizon A and B irrigated with, United sugar industries effluents. Horizon A and B of FFC Machhi Goth Evaporation pond sediments and reference soil samples from Ghotki and Sadiqabad irrigated with canal water. The values in Parenthesis indicate Confidence limit at 95% level

	U.S.Mill Horizon A	U.S.Mill Horizon B	FFCMachi Goth Horizon A+B	Ref.soil SDK Horizon A	Ref.soil SDK Horizon B	Ref.soil Ghotki Horizon A	Ref.soil Ghotki Horizon B
	Mean	Mean	Mean	Mean	Mean	Mean	Mean
Gly	440 (30)	400 (30)	425 (197)	345 (15)	305 (15)	305 (15)	315 (15)
Ala	705 (106)	700 (60)	775 (167)	555 (15)	535 (14)	640 (15)	590 (30)
Val	675 (15)	655 (15)	660 (30)	655 (45)	535 (45)	495 (15)	BDL
Leu	205 (15)	155 (15)	155 (76)	145 (15)	55 (15)	105 (15)	85 (15)
Ser	225 (16)	125 (15)	165 (197)	175 (15)	85 (15)	95 (15)	45 (15)
Thr	315 (15)	295 (16)	295 (136)	195 (15)	145 (14)	205 (14)	155 (14)
Ile	75 (15)	95 (15)	75 (15)	35 (15)	25 (15)	45 (15)	25 (16)
Cys	65 (17)	10 (12)	75 (45)	45 (16)	35 (15)	45 (15)	35 (15)
Met	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Asp	1175 (136)	1000 (152)	1040 (121)	690 (30)	540 (30)	885 (15)	775 (30)
Asn	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Glu	935 (15)	640 (30)	800 (395)	640 (30)	430 (31)	705 (15)	630 (15)
Gln	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Pro	105 (15)	125 (15)	125 (45)	105 (15)	75 (15)	95 (15)	65 (15)
Lys	325 (15)	300 (30)	305 (197)	240 (30)	125 (15)	295 (14)	135 (15)
His	65 (16)	55 (15)	65 (15)	45 (15)	25 (14)	85 (15)	55 (15)
Phe	75 (15)	BDL	70 (10)	35 (15)	BDL	20 (0)	BDL
Tyr	65 (15)	75 (15)	90 (12)	55 (16)	35 (15)	65 (15)	25 (15)
Trp	BDL	BDL	BDL	BDL	BDL	BDL	BDL

Table-5: (a) one way ANOVA comparison of amino acids, TN and OC among JDW sugar mill effluent irrigated and reference soil samples irrigated with fresh canal water (SDK, Sona Mahar)

Amino acids	Statistics	Sum of Squares	df	Mean Square	F	Significance
Gly	Between Groups	139141.4	1	139141.4	1.3	
	Within Groups	22958.6	5	4591.7		
	Total	162100	6			
Ala	Between Groups	83684.2	1	83684.2	22.4	Significance
	Within Groups	18598.1	5	3719.7		
	Total	102282.3	6			
Val	Between Groups	61570.1	1	61570.1	6.9	Significance
	Within Groups	44330.3	5	8866.5		
	Total	105900.4	6			
Leu	Between Groups	2570.5	1	2570.5	1.30	
	Within Groups	15930.2	5	.3186		
	Total	18500.4	6			
Se	Between Groups	2004.2	1	2004.2	2.5	
	Within Groups	3595.2	5	779.1		
	Total	5599.4	6			
Thr	Between Groups	52427.6	1	52427.6	4.7	
	Within Groups	55572	5	11114.4		
	Total	107999.6	6			
Ile	Between Groups	8724.3	1	8724.3	5.7	
	Within Groups	7575.1	5	1515.1		
	Total	16299.4	6			
Cys	Between Groups	2050.3	1	2050.3	. 6.6	.
	Within Groups	1550.1	5	310		
	Total	3600.4	6			
Asp	Between Groups	408748.5	1	408748.5	5.4	
	Within Groups	375551.0	5	.75110.2		
	Total	784299.5	6			

Table-5: (b) one way ANOVA comparison of amino acids, TN and OC among JDW sugar mill effluent irrigated and reference soil samples irrigated with fresh canal water (SDK, Sona Mahar)

Amino acids	Statistics	Sum of Squares	df	Mean Square	F	Significance
Glu	Between Groups	188697.1	1	188697.1	5.5	
	Within Groups	169002.3	5	33800		
	Total	357699.4	6			
Pro	Between Groups	1890.3	1	1890.3	3.7	
	Within Groups	2510.1	5	521		
	Total	4400.4	6			
Lys	Between Groups	132977.4	1	132977.4	3.7	
	Within Groups	7822.1	5	1564.4		
	Total	140799.5	6			
His	Between Groups	3399.0	1	3399.0	3.5	
	Within Groups	4770.2	5	594.1		
	Total	8169.2	6			

Table-5(b) continue

Phe	**Between Groups**	**6964.2**	**1**	**6964.2**	**3.4**	**.**
	Within Groups	**10135.7**	**5**	**2027.1**		
	Total	**17099.9**	**6**			
Tyr	**Between Groups**	**1467.8**	**1**	**1467.8**	**6.6**	**.**
	Within Groups	**1332.5**	**5**	**222.1**		
	Total	**2800.3**	**6**			
TN	**Between Groups**	**186468.3**	**1**	**186468.3**	**2.7**	
	Within Groups	**341231.2**	**5**	**682.3**		
	Total	**527699.5**	**6**			
O.C	**Between Groups**	**236834.2**	**1**	**236834.2**	**1.4**	
	Within Groups	**570465.2**	**5**	**114093**		
	Total	**807299.4**	**6**			

Table-6: Coefficient of Correlation (r) of reference Soil Samples SDK (Sona Mahar) among amino acids, TN and OC.

	Gly	**Ala**	**Val**	**Leu**	**Ser**	**Thr**	**Ile**	**Cys**	**Asp**	**Glu**	**Pro**	**Lys**	**His**	**Phe**	**Tyr**	**TN**	**O.C**
Gly	**1**																
Ala	**0.97**	**1**															
Val	**0.99**	**0.93**	**1**														
Leu	**0.99**	**0.96**	**0.85**	**1**													
Ser	**0.91**	**0.95**	**0.85**	**0.98**	**1**												
Thr	**0.87**	**0.96**	**0.98**	**0.99**	**0.98**	**1**											
Ile	**0.85**	**0.95**	**0.97**	**0.78**	**0.78**	**0.83**	**1**										
Cys	**0.85**	**0.93**	**0.98**	**0.78**	**0.77**	**0.83**	**0.99**	**1**									
Asp	**0.92**	**0.9**	**0.99**	**0.99**	**0.99**	**0.99**	**0.79**	**0.79**	**1**								
Glu	**0.9**	**0.93**	**0.97**	**0.98**	**0.99**	**0.99**	**0.77**	**0.77**	**0.99**	**1**							
Pro	**0.93**	**0.98**	**0.99**	**0.97**	**0.97**	**0.98**	**0.89**	**0.89**	**0.98**	**0.97**	**1**						
Lys	**0.97**	**0.94**	**0.97**	**0.98**	**0.98**	**0.99**	**0.79**	**0.79**	**0.99**	**0.99**	**0.98**	**1**					
His	**0.96**	**0.99**	**0.98**	**0.99**	**0.93**	**0.96**	**0.94**	**0.94**	**0.94**	**0.93**	**0.97**	**0.94**	**1**				
Phe	**0.98**	**0.98**	**0.95**	**0.91**	**0.99**	**0.98**	**0.99**	**0.99**	**0.99**	**0.99**	**0.99**	**0.99**	**0.99**	**1**			
Tyr	**0.97**	**0.99**	**0.98**	**0.93**	**0.93**	**0.96**	**0.94**	**0.94**	**0.94**	**0.93**	**0.98**	**0.94**	**0.99**	**0.99**	**1**		
TN	**0.93**	**0.70**	**0.53**	**0.41**	**0.41**	**0.49**	**0.89**	**0.89**	**0.43**	**0.40**	**0.61***	**0.43**	**0.70***	**0.1***	**0.70**	**1**	
O.C	**0.12**	**0.34**	**0.12**	**0.005**	**0.005**	**0.08**	**0.62***	**0.62**	**0.01***	**0.02**	**0.20**	**0.01***	**0.34**	**0.1**	**0.34**	**0.90**	**1**

* Values indicate negative correlation

The total nitrogen calculated by mico Kjhaldal method was within the range 290 - 1120 mg/Kg from the soils receiving industrial effluents as compared to 360 - 700 mg Kg^{-1} for the reference soil samples using fresh water for cultivation. Similarly organic carbon was calculated within the range 720 - 1680 mg Kg^{-1} for the soil receiving industrial effluents as compared to 490 - 1120 mg Kg^{-1} for soil receiving only fresh water (reference soil). The ratio of organic carbon to total nitrogen (C/N) calculated varied within 1.3 - 3.3 at the soils receiving industrial effluents as compared1.3 - 1.8 observed at reference soils. The observed ratio was lower than reported from cultivated soils in Lowa, USA (6.5 - 14.7) [29] and Gwynedd Bangor, UK (10.8- 13.0) [32]. It may be due to high temperature (up to 45^0C) prevalent within the area may have accelerates microbiological decomposition of organic compounds present in the soil and decrease in C/N ratio.

Coefficient of correlation between amino acids, total nitrogen and organic carbon was calculated for the soil receiving JDW sugar mill and reference soils from SDK. A positive correlation was observed for total nitrogen against Gly (0.68), Ala (0.13), Leu (0.71), Thr (0.15), Cys (0.11), Asp (0.46), Glu (0.75), Pro (0.24), Lys (0.32), His (0.51), Phe (0.81), Tyr (0.67) and organic carbon against Gly (0.67), Ala (0.15), Val (0.11), Ser (0.77), Thr (0.22), Asp (0.46), Glu (0.69), Pro (0.23), Lys (0.25), His (0.49), Phe (0.75), Tyr (0.65) and TN (0.99) for the soil using the effluents of JDW sugar mill (Table-6). Similarly the reference soil analyzed from SDK indicated a positive correlation of total nitrogen against Gly (0.93), Ala (0.70),Val (0.53), Leu (0.41), Ser (0.41), Thr (0.49), Ile (0.89), Asp (0.43), Glu (0.40), Lys (0.43), Tyr (0.70) and organic carbon with Gly (0.12), Ala (0.34), Val (0.12), Cys (0.62), Pro (0.20), His (0.34), Phe (0.1), Thr (0.34) and TN (0.90) (Table-7).

Experimental

The chemical deirvatization of amino acids with FAA and ECF was examined, followed by solvent extraction and GC elution and separation from GC column HP-5(30 m × 0.32 mm id) at the conditions described in the experimental. All the amino acids eluted and separated completely within 10.5 min. The identification of each amino acid was carried out by comparing the retention time with standards and by spiking each of the amino acid in sequence. Repeatability of the separation was examined at the concentration of 6.0 µg/ml each of amino acid in term of retention time and peak height (n=6) and the relative standard deviation (RSD) was observed in the range of 1.6-2.1% and 1.4-1.9% respectively.

The soil samples from 15 locations were collected from Districts Rahimyar Khan (Punjab) and Ghotki (Sindh) Pakistan during 2010, 2011. The sampling stations were using effluents of sugar, edible oil and fertilizer industries for irrigation purposes from 4 to 15 years. The samples also include bottom sediments of evaporation ponds from two locations used for collection of effluents of sugar mill and fertilizer industry. The soil samples (4) within the vicinity using the fresh water (canal irrigated) were also collected as reference for comparison.

Description of Sampling Area

Sampling locations were selected five from the agricultural lands receiving effluent of Jamaluddin Wali (JDW) sugar mill, Rungpur; two samples receiving effluents of United Sugar Mill (USM), Bakhshabad, three receiving effluents of Edible oil (oil andghee) industries Bahadur pur, and a sample from evaporation pond Fauji Fertilizer Company, Machi Goth, Tehsil Sadiqabad (SDK), District Rahimyar Khan (Punjab). Three sampling locations were selected from agricultural lands receiving effluents of Ghulam Muhammad Mahar Sugar Mills (GMMSM), Wallu Mahar and a sample of bottom sediments of evaporation pond receiving effluents of Ghotki Sugars Mills, Taluka Ghotki, District, Ghotki (Sindh), Pakistan was also collected. Four reference soil samples two each from Tehsil Sadiqabad (SDK) and Tehsil Ghotki using fresh canal water for irrigation.

The agricultural lands selected for sampling are located within canal irrigated zones. The effluents are used during water scarcity for irrigation during winter. The land is used for cultivation of cotton and wheat under rotation. The canal water carries silt which is deposited in the field. The industrial effluent added to the land is mostly dark in color. The temperature within the study area varies from 4…45^{0}C. The soil in the area is clay, silty and loamy.

Soil sampling

The soil samples were collected during December March 2010 - 2011 when effluents were being used for irrigation. The sampling scheme was repeated twice. Each plot of about 220 sq feet was selected. Composite sampling scheme was used, where four to six samples from 0-15 cm depth at a distance of about 30 - 50 feet were collected and mixed together and a sample of 0.5 kg was collected from each location and labeled as "A". Similarly another sample was also collected from the same location at the depth of 15 -30 cm and was labeled as "B" sample.

The samples were collected with clean stainless steel circular agar (3.6 cm diameter×30cm length). All the soil inside the agar was collected and mixed together. The composite sample was transferred to polyethylene bag, kept cool and transported to the laboratory. The samples were air dried and sieved through 2 mm sieve to remove stones, crop materials and roots. A portion from sieved material (5g) was heated at 105^{0}C in oven for 6h to determine the moisture contents.

Sample Analysis

The corresponding to dried soil (20 g) was added deionized distilled water (60 ml) and was shaken on mechanical shaker for 1h. The soil was allowed to settle and pH was measured with calibrated Orion 420 A pH meter (Orion Research, Inc. Boston, MA USA) combined with glass electrode and reference internal electrode. Total Nitrogen (TN) was determined by micro-Kjeldal method [33]. The organic carbon (OC) was calculated from COD as reported [34, 35].

Analysis of Amino Acids

Dried soil sample 1g was added 20 ml of 6 N hydrochloric acid and the contents were heated in screw capped sample vial at 110^{0}C for 24h. The mixture was cooled and centrifuged for 20 min at 3000 g. The clean supernatant layer was separated and the residue was added deionized water (5ml) and the contents were mixed well. The mixture was again centrifuged for 20 min. The solvent from combined clear solution was evaporated gently under nitrogen atmosphere. The residue was dissolved in water and the volume was made up to 15 ml. The solution (1ml) was taken and GC derivatization procedure was followed as reported [10].

The acid hydrolyzed aqueous extract of soil containing a mixture of amino acids was added to 0.5 ml ammonium acetate buffer pH 7 and 0.5 ml of trifluoroacetylacetone (FAA) (2% v/v in methanol). The contents were heated on water bath for 20 min. The mixture was then added to ethyl chloroformate (ECF) (0.5 ml) and carbonate buffer pH 9 (0.5 ml). The mixture was sonicated at room temperature (30^{0}C) for 15 min. Chloroform (0.5 ml) was added and the contents were mixed well. The layers were allowed to separate and aliquot of the organic layer was transferred to screw capped sample vial (1 ml). The solution (1.0 µl) was injected in GC with split

ratio 10:1 v/v on the column HP5 (30 m×0.32 mm id) with film thickness 0.25 µm at column temperature 100^0C for 2 min, followed by heating rate of 20^0C/min up to 250^0C. The nitrogen flow rate was 3 ml/min. The injector and detector temperatures were 270 and 280^0C respectively. The flow rates for FID were fixed for nitrogen as make up gas 45 ml/min, hydrogen 40 ml/min and air 450 ml/min. The quantification was made from linear regression equation

$$Y= ax + b$$

Analysis of amino acids from soil sample spiked with amino acids standard

Dried soil sample (1g) was treated as above procedure for amino acids analysis. Two portions of 1 ml each from a sample were taken. A portion was added to the solution of a mixture of amino acids (0.5 ml) containing 10 µg/ml each, and both the portions were processed as derivatizing procedure for amino acids. The quantification was carried out from external calibration curve and from the increase in response with added standards.

Chemicals and Solutions

The compound glycine (Gly), L-alanine (ala), L-valine (Val), L-phenylalanine (Phe), tryptophan (Trp), (Sigma Louis, USA), tyrosine (Tyr), serine (Ser), leucine (Leu), isoleucine (Ile), methionine (Met), threonine (Thr), proline (Pro), (Sigma, Deisenhofen, Germany), glutamic acid (Glu), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), lysine (Lys) and histadine (His) (Sigma, Gmbh, Germany), trifloroacetylacetone (FAA) and ethyl chloroformate (ECF) (Fluka, Bucks, Switzerland), methanol (Rdh, Chemical Co. Spring Valley, CA, USA) were used. Guaranteed reagent grade hydrochloric acid (37%), potassium chloride, acetic acid, sodium acetate, sodium tetraborate, boric acid, sodium bicarbonate, ammonium chloride and ammonia solution were from Merck, Darmstadt, Germany.

Stock solutions of amino acids containing 1000 µg/ml for each amino acid were prepared in methanol and water. Further solutions were prepared by appropriate dilution. Buffer solutions (0.1M) between pH 3-10 at unit interval were prepared from the following: acetic acid-sodium acetate (pH 3...6), ammonium acetate (pH 7), boric acid-sodium tetraborate (pH 7.5…8.5), sodium bicarbonate-sodium carbonate (pH 9) and ammonium chloride-ammonia solution (pH 10).

Equipment

GC studies was carried out on an Agilent model 6890 network GC system, connected with flame ionization detector (FID) and split injector (Agilent Technologies, Sanata, Clara, CA,USA) hydrogen generator (Parker Balston, Analytical Gas System, H_2-90, Parker Hannifin Havershill, MA, USA) and pure nitrogen (British Oxygen Company (BOC) Karachi, Pakistan), computer with Chemistation software controlled the gas chromatograph. Capillary column HP-5 (30 m ×0.32 mm id) with film thickness 0.25 µm (J and W Scientific GC column, Wilmington, NC, USA) was used throughout the study.

Conclusion

The soil samples receiving effluents for cultivation from sugar mill, oil and ghee industry and fertilizer factory were analyzed for acid hydrolyzed amino acids, total nitrogen, organic carbon and pH. Amino acids were analyzed by GC using FAA and ECF as derivatizing reagents. The soil receiving industrial effluent and reference soil collected from Tahsil Ghotki indicated highest concentration of the amino acids Asp > Glu > Ala > Val at level-A, but the reference samples collected from Taluka Sadiqabad indicated slightly different pattern with Asp > Val > Glu > Ala at A level. The observed relative concentration of amino acids in the soil receiving the effluents of JDW sugar mill at A level were with following decreasing order: Asp > Glu > Ala > Val > Gly > Lys > Thr > Leu > Ser > Pro > Ile > Phe > His > Cys. Similarly the soil receiving effluents of edible oil mill (oil and ghee) indicated the amino acids in following decreasing order: Asp> Glu>Ala>Val>Lys>Thr>Leu>Ser>Pro>His>Tyr>Ile >Cys>Phe.The relative amounts of amino acids was somewhat differed from reference samples from the same area using fresh water with following order: Asp > Val > Val > Glu > Ala > Gly > Thr > Lys > Leu > Pro > His > Ser > Cys > Tyr > Ile.

The concentration of amino acids detected were observed in the range for the soil receiving JDW Sugar mill effluent within 66-1074 mg kg^{-1} and oil and ghee effluents within 60-1006 mg kg^{-1} as compared to reference samples at the same level (A) within 35-690 mg kg^{-1}. The average results of analysis of soil receiving effluents of JDW sugar mill and oil and ghee effluents were compared with the average results of reference soil samples from Sadiqabad (SDK) for amino acids. The test for one

way analysis of variance (ANOVA) was applied and a significant difference was indicated at 95% confidence level between soil receiving the effluents and reference soil samples for Ala and Val

Amino acids contents, total nitrogen and organic carbon in soil receiving industrial effluents were higher than the soil cultivated on fresh water.

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