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Abiotechnology of *Indigofera tinctoria* L. on the Saline Land of Aral Sea Basin and Producing of the Natural Plant Indigo Pigment for the Industry

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Abstract: The possibility of cultivation of *Indigofera tinctoria* L. plants in ecologically degraded and saline lands of the Aral Sea Basin was studied. A new salt-resistant *Indigofera* plant variety "Feruz-1" was producing by the classic methods of genetics and modern biotechnology.Agro-technology of cultivation as main and secondary culture after wheat was developed and recommended to the farmers of the country.The biotechnology of natural plant pigment indigo extraction has been developed and was recommended for using in different fields of industry: textile, pharmacy-pharmacology, perfume-cosmetic, architectural-decorative and food industry. Quantitative and qualitative HPLC (high pressure liquid chromatography) method of determination of plant natural indigo has been also developed.

Key words: Indigo dye, Aral Sea Basin, salt-resistance, cultivation, HPLC.

1. Introduction

Solutions to many global ecological problems require not only one scientific but integrated, i.e., interdisciplinary approaches. The most important and critical among those are global climate change, water and air pollution of the planet, population upsurge, exhaustion of energy resources, conservation of biological variety of gene pool and ecosystem, struggle against degradation and desertification, salinization of soil, etc..

For example, if deterioration of soil fertility happens as a result of degradation or salinization then surely it results in reduced crop yield of agricultural fields, which leads to the food shortage associated with hunger and illnesses. This, in turn, leads to social perturbation, conflicts, and finally to the loss of sustainability of development of society. Therefore, joint efforts and cooperation of scientists with different profiles, specialists from different fields of science, with different approaches and methods of investigation are required. This approach is currently often wider and successfully taken in various projects of UNESCO (United Nations Educational, Scientific and Cultural Organization), many other International Projects, Programs and etc..

Due to complete failure to integrate or lack of effective coordination of, interdisciplinary approach in the last few decades, we allowed initiation of many above mentioned global problems, solutions of which, now, certainly will be very difficult and unexpectedly high cost for all of us.

As a result of desiccation of the Aral Sea, more than five million hectares of degraded and saline land has

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appeared. This is much more than all irrigated lands occupied in agriculture of the Republic of Uzbekistan, i.e., 4.3 million ha, with population of more than 30 million people [1-3].

At the moment, annually 150,000 tons of toxic mixture of deposited salt and pesticides used for decades in cotton growing agriculture are being scattered to thousands of kilometers, polluting water, air, soil, plant and animal world [4-6].

In this respect, in solution of such a problem, one of the ecologically reasonable and not so expensive methods is to create "Green Carpet", with testing from the world botanical collection or by creating new salt-resistant plants [7-9]. Furthermore, it is preferred to develop not soexpensive biotechnologies to produce economically viable natural substances for various fields of industry (United Nations Development Program's Project: "Shelterbelt"around the Aral Sea Region in Uzbekistan, 1995; UNESCO's Project: "*Indigofera* plantations for the Aral Sea Basin", A. Ergashev, Tashkent, 2005).

The genus *Indigofera*, the third largest in the family *Leguminosae*, consists of almost 800 species. These species usually can grow on land between sea level at 1,650 m. Over 600 types can be found in Africa, nearly 200 in Asia, more than 80 in America, and another 50-60 in Australia [10].

However, the most widely used types in obtaining natural plant dye indigo in the world are found mainly in tropical and subtropical zones. *Indigofera tinctoria* L. (= I. sumatrana Gaertn) is one of these varieties. In optimum soil-climate conditions, the height of plant habitus can be reached up to 1.5-2 m. *Indigofera* plants have been used since the ancient Egypt pharaoh Tutankhamen times as dye sours. In Tibet and Indo-Chinese territories, it was used by medical folks as the source of substances to cure dog and snake bites, antibacterial and antifungal agent, to cure toxicities of liver, antidepressant, and anticancer drug [10, 11]. Water broth of roots of this plant is used to treat typhoid fever, cholera, etc.. Recently, the usage of this plant in treatment of serious infectious illnesses of genital organs, renal and nervous diseases has also been reported [11]. Therefore, year by year, the demand on this natural dye indigo has been increasing (Table 1) and its price in Europe is fluctuated between 80 euro·kg^{-1} to 240 euro·kg^{-1}.

According to geo-botanic investigations shown in Fig. 1, *Indigofera tinctoria* [10] were not previously grown and/or could not be cultivated in saline soil-climate zones of the Aral Sea region, without using of genetic approach proposed by our team [12].

2. Materials and Methods

Classic genetic approaches, including individual selection of unique salt resistant genotypes as well as modern methods of biotechnology, genetic engineering, aerospace photography, physicochemical analysis, soil microbiology, marketing studies, etc., were used in our research to promote the variety of *Indigofera tinctoria* for the conditions of the Aral Sea region.

Main field investigations were carried out directly on heavily salted field of experimental farm of the UrSU (Urgench State University) named after Al-Khorezmi (Fig. 2). Due to the lack of special seeding machine for sowing of *Indigofera* seeds, their sowing and some other agro-technical activities on growth care of plants were carried out by hand.

The use of indigenous, i.e., wild soil strains of *Agrobacteria*, associated with many other plant root sphere and its capability to fix atmospheric nitrogen

Table 1 Annual requirement in the world market: indigo/natural indigo (in tons).

Year	2005	2006	2007	2008	2009	2010	2011	2012
World requirement	18,848	19,508	20,191	20,897	21,629	22,386	23,169	23,980
In Europe	2,827	2,926	3,028	3,134	3,244	3,374	3,509	3,649
Natural indigo demand	2,309	2,390	2,474	2,560	2,650	2,743	2,839	2,938
Natural indigo demand in Europe	349	361	373	386	400	416	433	450

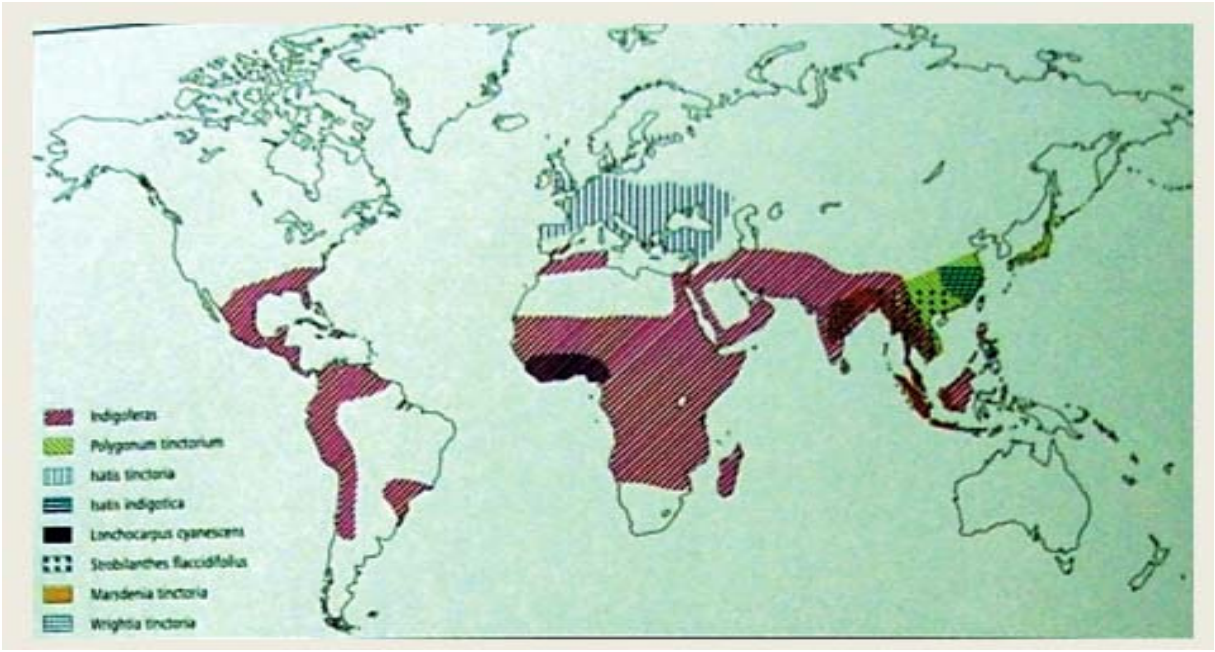

Fig. 1 Map of the geo-botanicareal of distribution of Indigo-producing plants.

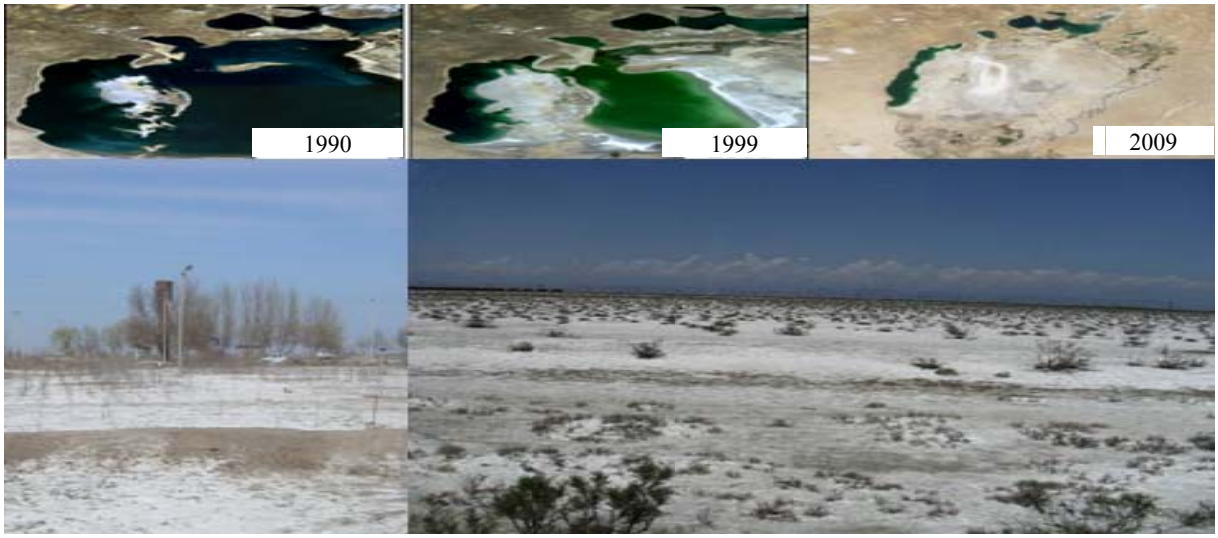

Fig. 2 Aerospace photos of NASA&UN-SPIDER (United Nations Space-based Information for Disaster Management and Emergency Response) taken in different years of Aral Sea and deeply salted experimental field of UrSU (bottom).

even in deeply saline soils were the most important instrument [12].

As is generally known, owing to the presence of gigantic Ti-plasmids with atmospheric nitrogen fixing genes, i.e., Nif-operon in bacterial cells, bacterium entering into partnership with the cells of host plant which starts to provide them the required molecules of nitrogen. The in turn starts to provide the bacterial cells the required products of photosynthesis.

Figs. 3 and 4, a scheme explaining the formation of root tuber and organization of nod-gene regulation of some nitrogen fixing bacteria [18]. Upper part of Fig. 4—localization of functional segments in mega plasmid, in the middle—structure of nod-, hsn- and efn- regions. Black bold arrows indicate location of promoters and direction of transcription. In principle, such forms of co-existence, i.e., symbiosis between two different varieties in nature are widespread, especially when a variety itself needs in adaptation to a new ecological place or area.

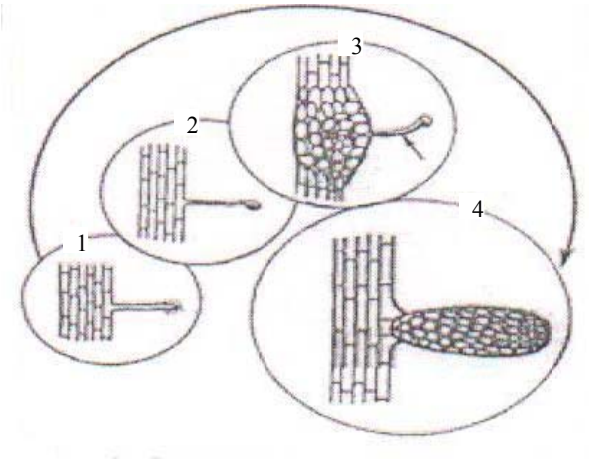

Fig. 3 A scheme of formation of root nodules.1—attachment of bacteria, 2—winding of root hairs, 3—formation of infectious strand, 4—mature root tuber.

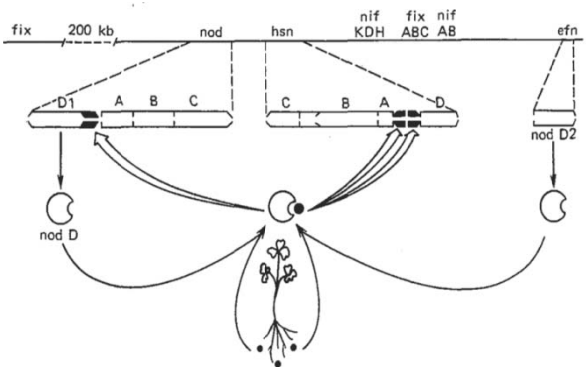

Fig. 4 Organization of nod-gene regulation in R. melilioti.

The widespread natural phenomena of Crone galls of trees (Ti-plasmids as a "Horizontal Heredity" or very old rudiment of the transgenic mechanism of the first Cancer Cells in evolution. They are used of atmospheric N for the synthetizing of amino acids of proteins) and integration of Ti-plasmids DNA with DNA of plant cells was underlined in theoretical basis of our research [12].

3. Results and Discussion

A five thousand seeds of *Indigofera tinctoria* L. kindly provided by the Institute of Botany Academy of Sciences of Uzbekistan from its world collection were tested on saline soil of the experimental farm of the UrSU, the region nearer and typical to climatic conditions of the dried Aral Sea bottom.

Seed collected from those single, unique genotypes (Fig. 5) were sowed further for propagation in breeding nursery of private owned farm of "Indigo Jonibek" in Kibray District of Tashkent Region established for salt-resistant variety of *Indigofera* "Feruz-1" (which is registered by the State Commission of the Republic of Uzbekistan as a new variety and recommended for the cultivation in the all the regions of the country).

Fig. 5 The unique genotype of the plant of *Indigofera*, with high capability of association with indigenous strains of nitrogen fixing agrobacteria on saline soils of the Aral Sea Basin.

The green plant biomass cultivated in saline soil field conditions was used for the extraction of natural plant pigment indigo (Fig. 6). And after fermentation of green biomass in water for 24 h, all the liquid was transferred into the second lower container for oxygen enrichment. After appearance of dark blue color, the liquid was filtered through the cloth filter as in traditional folk method for extracting dairy products (Fig. 7).

External appearance and color of the indigo dye is determined by visual inspection, and quantitative determination of the indigo dye in the substance is carried out by HPLC (high pressure liquid chromatography) method (Figs. 8 and 9).

Fig. 6 Harvesting of green biomass of the *Indigofera* plant "Feruz-1" variety, shown a significant improving of soil fertility.

Fig. 7 Dark blue liquid with indigo is filtered through the cloth by folk method.

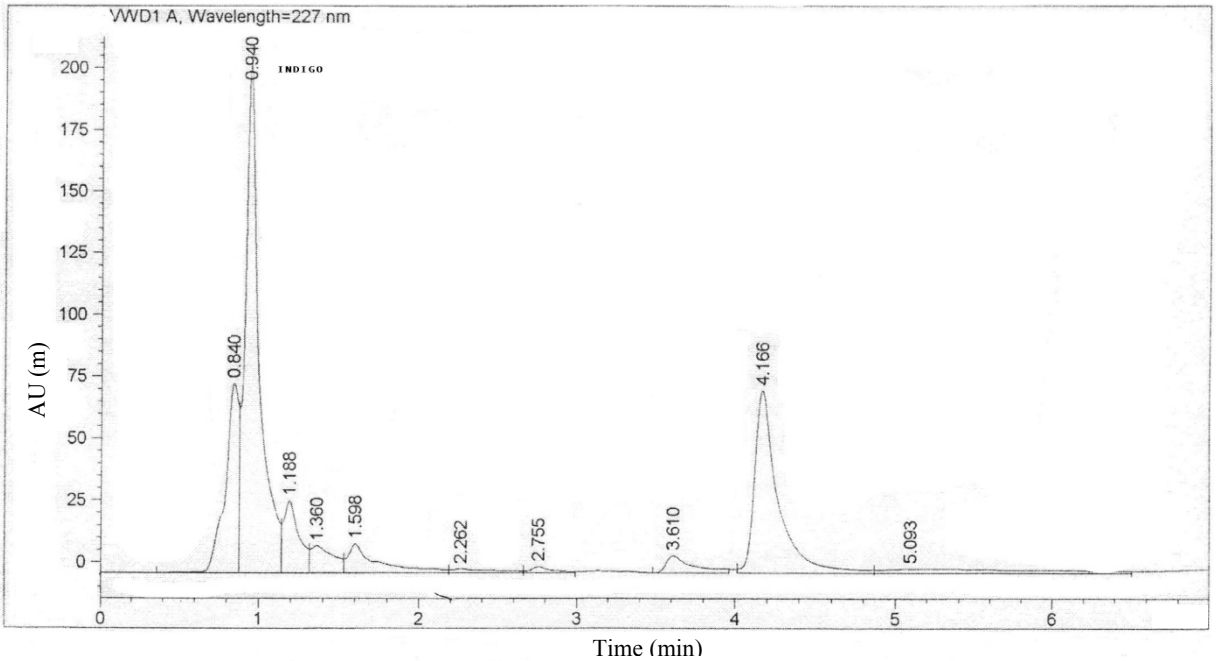

Fig. 8 HPLC chromatogram of natural indigo isolated from "Feruz-1" indigo plant variety.

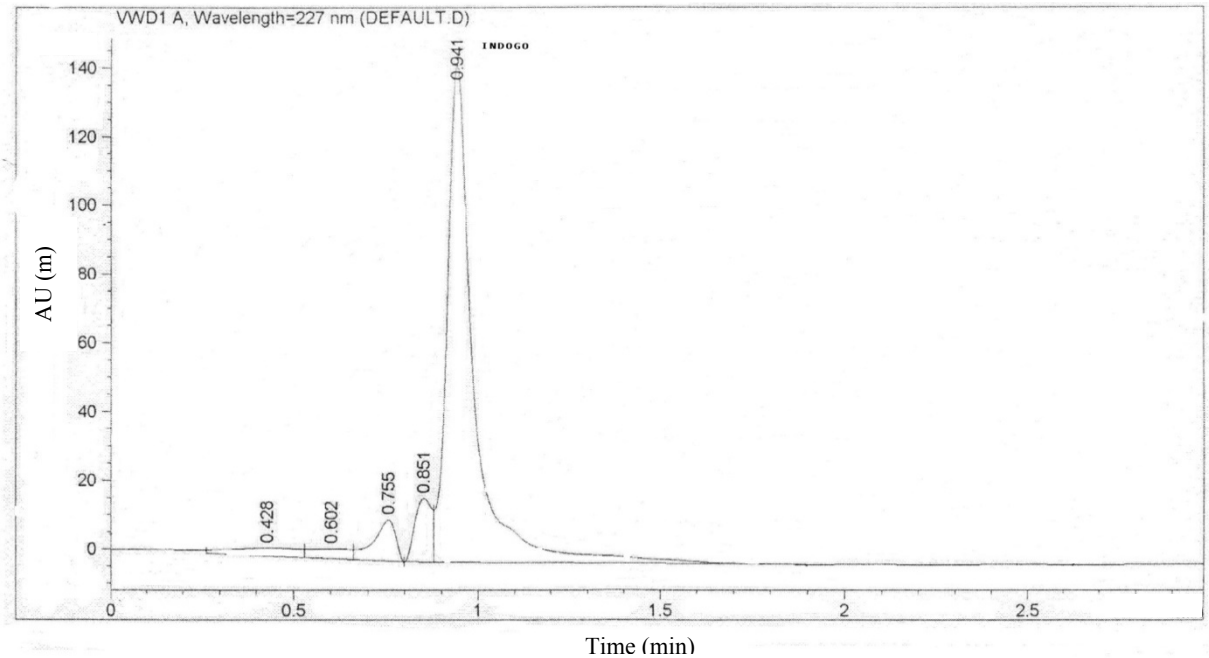

Fig. 9 HPLC chromatogram of synthetic indigo (standard), manufactured in Germany (Farbmuhle, D-88317 Aichstetten, Kremer pigment, 23,100, Indican Hitenblou).

Agilent liquid chromatograph supplied with UVDAD (ultra violet diode array detector) detection with variable wavelength was used. Column was ZORBAX SBC18 with dimensions of 3.0 mm × 150 mm × 3.5 mm. Isocratic elution mode, with elution speed of 1 mL·min^{-1}, was used to carry out the chromatographic separation.

Indigo dye in an amount of 0.0250 g was dissolved in 25 mL of acetonitrile to make 1 mL·min^{-1} and was injected from 1 μL to 10 μL. The obtained solution was filtered and centrifuged at 10,000 rpm for 5 min or was filtered through Millipore filter of 0.22 μL

before chromatography analysis. Mobile phase consisted of acetonitrile: 0.01 M hydrochloric acid:glacial acetic acid:water in the ratio of 50:34:1:15.

Speed of elution is 1 $mL \cdot min^{-1}$ while wavelength is detected at 227 nm. Recording integrator (Shimadzu Chromatopac C-R2AX, Japan) was used to quantity the area of peaks. Retention time of the main peak is 0.9-2.5 min (depending on the column used in the experiment). Per cent content of the indigo dye in the sample was determined relative to the peak area of the standard indigo sample. It was not less than 45%-50%.

Synthetic indigo was used as standard for the quantitative determination of natural indigo isolated from the plant. Figs. 8 and 9 show the chromatogram of the natural and standard samples of synthetic indigo pigment. The peak area at 0.941 min was adopted as 100% of substance as the other peaks corresponding to by-products were absent in the chromatogram. The percent content of natural indigo dye (Fig. 8) in the sample was determined relative to the peak area of the standard synthetic sample of indigo (Figs. 9 and 10). It was at least 45%-50%.

Further, synthetic indigo was chromatographed by HPLC method on PerkinElmer Series-200 with auto sampler. Identical chromatograms were obtained and 2D image, adsorption, and image of thin-layer chromatography were obtained using the program Total Chrome Navigator. So, it was concluded that HPLC can be used for qualitative characteristics and quantitative determination of natural indigo as modern, widely available analytical method for quality control of products.

3.1 Determination of Wet Content

The wet content was determined according to [19]. Exact sample of the substance was put into a pre-dried weighing bottle and was dried till the constant mass. Weighing bottle with lid was put into desiccator to dry

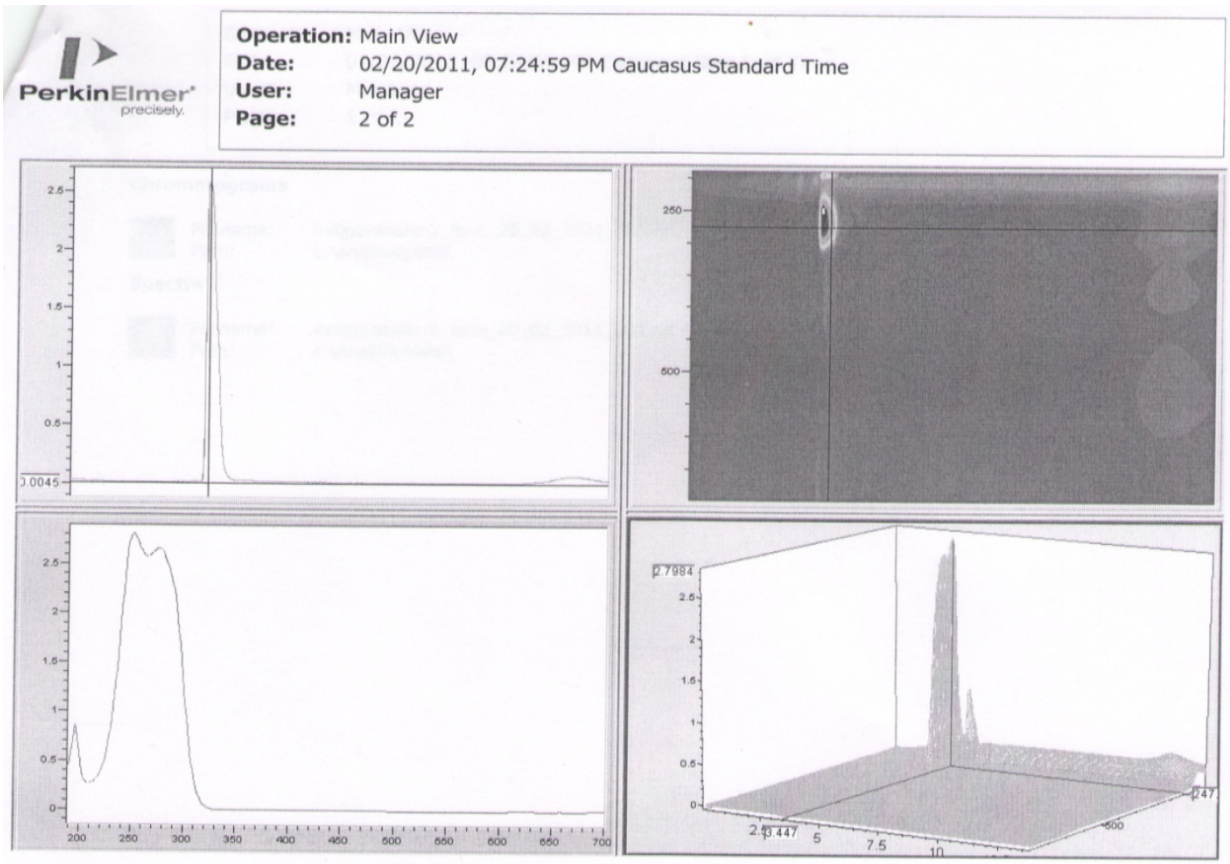

Fig. 10 3D spectrum of synthetic indigo.

for 50 min and then was weighed. First weigh was done after 2 h after drying, and further weighing was carried out after 1 h till the constant mass was reached.

3.2 Determination of Apparent Density

The apparent density of indigo dye was determined in a container having the volume of 0.1 cm^3. In the beginning, the mass of container was determined. Then container with indigo was gradually put. The height of fall of indigo must not exceed 250 mm. After filling the container, the surface of indigo was aligned with strip and pieces were removed that hinder the free movement of the strip. After that, the full container was weighed. Iterative determination was performed using second part of the test, repeating all the operations.

The apparent density of indigo in recalculation to dry substance Z in $g \cdot cm^{-3}$ is calculated according to Eq. (1):

$$Z = \frac{m_2 - m_1}{V} \cdot \frac{100 - M}{100} \quad (1)$$

where, m_1—the mass of empty container (g); m_2—the mass of container with indigo (g); V—the volume of container (cm^3); M—the content of total wet in indigo (%).

An average arithmetical data from two calculations was taken for the result. If qualification of indigo is not included into the protocol, the apparent density is expressed in recalculation to dry substance. If the apparent density is required at the time of sale, the coefficient $\frac{100 - M}{100}$ can be not included in above given calculation, and the result includes into a protocol with qualification "wet substance at the time of sale" or "at the time of obtaining".

4. Conclusions

Thus, practical usage of such unique plant dye—indigo goes with its roots far deep into centuries and antique civilization. However, in spite of many secrets of cultivation of plant, extraction of valuable natural pigment and many valuable drugs from green biomass for practical use have been lost. Even in our days, in theoretical and practical plan, they remain not elucidated till the last being property or art of famous national masters of India, China, Japan and others.

It was thought so far that such tropical cultivar as *Indigofera* was impossible to cultivate in arid conditions, all the more, in saline lands of the Aral Sea region in industrial scale.

By initiation of UNESCO Office in Tashkent "Uzindigo" project was organized in 2005 to study the possibility of cultivation and obtaining of tonnage biomass of *Indigofera tinctoria* in salted lands of the Aral Sea region, i.e., in Khoresm, Karakalpakstan and Navoiy Region of the Republic of Uzbekistan. *Indigofera tinctoria* L. belong to Leguminous family—half shrub-herbaceous plants and by the method of individual selection of unique genotypes a new "Feruz-1" variety was created. Because of developed symbiosis with indigenous nitrogen fixing tubercular bacteria, this variety can grow in quite high levels of saline lands of the Aral Sea region.

Now, due to support and co-financing by Small Grants Programs of the Global Environment Facility SGP GEF, UNDP, UNESCO Office in Uzbekistan the specialized seed farming was established. Also it was improved biotechnology for extracting of natural plant indigo from the green biomass in laboratories and field farms conditions.

Taking into account the increasing demand in the world for natural dyes, and also for there-cultivation of saline soils in Aral Sea Basin, our group of scientists in collaboration with UNESCO Chair at UrSU and ZEF Bonn University have developed cultivation of agro-technology of *Indigofera* plant on saline soils and producing of natural indigo dye to use in textile, pharmaceutical, perfumery-cosmetic, architectural-decorativeand other fields of industry (in European market the cost of 1 kg of natural indigo is from 80 Euro to 240 Euro).

Now, for a wide practical realization of such ecologically useful and economically beneficial cultivar as *Indigofera*, the highest obstacle would be

the lack of *Indigofera* seeds. Therefore, for interested farmers, scientists and others special seed farming of new "Feruz-1" variety is organized in Kibray District of Tashkent Region of Uzbekistan.

Thus, the following conclusions can be made for the first time as a result of carried out scientific investigations:

(1) The possibility of cultivation of *Indigofera* plant in ecologically degraded and saline lands of the Aral Sea region was shown for the first time [13-14];

(2) A new salt-resistant *Indigofera* variety "Feruz-1", capable to associate with indigenous strains of nitrogen fixing bacteria has been created and ecologically testedin different regions [15];

(3) Special seed farming of this variety of *Indigofera* has been established;

(4) Agro-technology of cultivation, as a main and secondary after the wheat culture, and biotechnology of extraction indigo pigment have been developed [16];

(5) Quantitative and qualitative methods of determination of indigo dye have been developed;

(6) A Biotechnology and pathway of biosynthesis of natural indigo pigment in vivo and in vitro conditions are studied;

(7) Educational-practical manual for farmers (in Uzbek, Russian, and English) has been published;

(8) A training center for farmers at Urgench State University has been established;

(9) More than 250 farmers from different regions of Uzbekistan were trained and conferred certificates;

(10) Marketing analysis on natural indigo has been carried out in Europe and the world [16];

(11) New salt-resistant variety of *Indigofera*"Feruz-1" has been included into the public register of cultivars authorized for cultivation in all regions of Uzbekistan (Protocol of the meeting of the State Commission of the Ministry of Agriculture and Water Resources of Uzbekistan on variety testing, November 30, 2011, Tashkent) [15].

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