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ISOLATION, IDENTIFICATION AND CONSERVATION OF POTENT HYDROLASE PRODUCER FROM DIFFERENT SOILS OF ODISHA, INDIA

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ABSTRACT

In this investigation, innate fungal species were isolated from soils of Odisha and screened for their hydrolytic potentials. Utmost numbers of fungi were isolated from botanical garden soil (3×10^8 CFU/g), Post Graduate Department of Botany, Utkal University, Odisha, India. Among 35 fungal taxa, *Aspergillus niger* was most predominant mycoflora followed by *Aspergillus terreus*. All fungal isolates were accredited to nine genera like *Alternaria, Curvularia, Fusarium* and *Mucor* represented two taxa, *Penicillium* by four taxa, *Rhizopus* by 3 taxa and *Trichoderma* represented one species. *Aspergillus* species were dominant groups that represented about 18 taxa. All isolates were screened for hydrolytic competence and most of the isolates biosynthesized one or more hydrolytic enzymes. *Aspergillus terreus* exhibited splendid diversity in biosynthesis of all investigated hydrolases. Hence, *A. terreus* could be a prospective contender in biosynthesizing high valued-low cost industrial enzymes from cheapest substrates addressing the welfare of mankind.

KEYWORDS: ***Aspergillus*, botanical garden, hydrolases, mycoflora, *Trichoderma*.**

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INTRODUCTION

Akin to all living beings, the soil heterotrophs- "fungi" represent a prime place in biodiversity that survive as saprotroph/ parasites/ biotroph or symbionts whereas numerous species also form mycorrhizal associations with plants or are plant pathogens[1]. They dwell in the majority part of this orb including soil, air and water unlike other microorganisms. Soil is called the elixir of life as the biology of soil is forever a fascinating and imperative milieu of study. Scores of advancement has been made in the universal understanding of the responsibility of soil organisms and biological purposes in upholding soil health. India is prosperous in fungal biodiversity which exceeds more than 27,000 species, the most widespread biotic community after insects[2]. A total of 32% from 205 new genera were accredited to C. V. Subramanian of the University of Madras which has been documented from India. Manoharachary *et al*.[3] has dignified the worth of fungal community by contributing 12 new genera, 60 new taxa and 500 novel fungi. Eleven novel instinctive fungal species were reported in the eighth edition of the Dictionary of the Fungi[4]. India is the one third contributor of a fungal miscellany of the entire orb. Out of the total 1.5 million fungi, about 50 % are epitomized and only 5–10 % of fungi are possible to be cultured *in vitro*. Besides to their praiseworthy significance, fungi execute the imperative job addressing the well beings of mankind in countless ways such as in industry, agriculture, pharmaceuticals, food industry, textiles, bioremediation, natural cycling, as biofertilizers, secondary metabolite production, industrial enzymes (e.g. amylases, cellulases, lipases, glucoamylases, pectinases, phosphatases and proteases). In present scenario, mycobiotechnology has been occupied the requisite position for betterment of humankind[5].

Only about 2 % of the world's microorganisms have been evaluated as enzyme sources. Bacteria produce 24 %, fungus 60 %, actinomycetes 2 %, yeasts 4 %, animals 6 % and plants 4 % of total large-scale enzyme biosynthesis[6]. Regardless of their significant magnificence, the taxonomy of these fungi is still not absolutely resolved and the species distribution in soil is not clearly reported. Nevertheless, many fungi particularly Aspergilli are acknowledged for their diverse groups of enzymes biosynthesis potentials; however the choice of a particular strain being a tedious task, especially when commercially proficient enzyme yields are to be attained. Fungal enzymes have been exploited in industries for decades owing to their multifaceted applications[7]; still there is an escalating hunt for the isolation and screening of novel fungal species. Though recombinant DNA technology has revolutionized research to achieve and manipulate microorganisms of commercial significance, nevertheless, conventional techniques are still employed. Keeping in view of the significant credentials of fungal enzymes, the present investigation was performed to isolate, enumerate, screen the instinctive fungal species and to explore their hydrolytic efficiency for the imminent prospective applications.

MATERIALS AND METHODS

(i) Chemicals and media

All the analytical grade chemicals and microbiological grade media components employed in the present investigation were purchased from Hi-Media Limited, SRL Pvt. Limited, Sigma Chemicals Co. (USA) and Merck India Limited (Mumbai, India).

(ii) Atmospheric condition and geographical location of the study site

Bhubaneswar is located between 21° 15' north latitude and 85° 15' longitude at an altitude of 45 meters above sea level with an area of 419 km^2 (162 sq miles). Geographically, Bhubaneswar is situated in the eastern coastal plains of Odisha, India and south-west of the

Mahanadi River (Fig 1). The city has a tropical savanna climate. The average temperature ranges between a minimum of around 12 °C (54 °F) in the winter to a maximum of 45 °C (113 °F) in summer. The average annual rainfall is 154 cm, most of which is recorded between June and October. Keonjhar is a land locked district of Odisha, India with an area of 8240 km^2. It is situated in the northern part of Odisha. It lies between 21°1' N and 22°10' N latitudes, between 85°11' E and 86 ° 22' E longitudes. It is situated at an altitude of 480 meter above sea level.

Map of Bhubaneswar

Figure 1
Location map of site selected for mycological study in Bhubaneswar, Odisha, India

(iii)Experimental field and soil sample collection

After selecting the spot, petite plants and other wreckage were removed ensuring the soil having very diminutive effect of rhizosphere and putrid bio-residues. The meadow was left under innate condition for about a fortnight before sampling. Surface soil about 2 cm profundities was removed to make the soil gratis of decomposing plant residues thus making natural soil available for the study purpose. About 1 Kg of soil samples were collected aseptically from each experimental field (5×5 m) of various areas of Bhubaneswar (Fig 1), Joda and Barbil of Keonjhar. All collected samples were brought to laboratory immediately and stored in refrigerator at 4 °C.

(iv) Isolation of potent microorganisms

The innate fungal strains were isolated from soil samples by serial dilution, pour-plate technique[8] and direct plate method[9]. The mixed cultures were obtained by pour-plating respective diluted samples on potato dextrose agar (PDA) and Czapek Dox agar (CZA) plates amended with sefixime (50 mg/100ml) under sterile conditions at 30 ± 1 ºC for 7 days. The isolates were stored at 4 ± 1 ºC until further use. All isolated fungi were identified as per Alexopoulos and Mims[10] and Watanabe[11] based on macro and microscopic characteristics. For macromorphological observations, fungi were pure cultured on CZA and PDA for 7 days at 30 °C in the dark to appraise growth on media with low a_w. Identification based on colony morphological study includes form, pattern, quantity of aerial hyphae, diameter, colony colour and texture, margin, elevation, colour of colony reverse, presence of exudates, organs formed and soluble pigment. For micromorphological observations, microscopic mounts were

performed in lactophenol-cotton blue solution by picking fungi from PDA plates and a drop of alcohol was added to remove air bubbles and excess conidia. Phase contrast microscopy (PCM) was performed to study the shape and size of head, vesicles, conidiophore and conidia, thickness of stipes and presence of phialides and metulas. The fungal isolates were also again characterized and identified by the National Center for Fungal Taxonomy, New Delhi, India.

(v) Preliminary screening of enzymes

All enzymatic assays were performed on pre-poured plates by point inoculation with mycelium/spores of fungal isolates as per Hankin and Anagnostakis[12] with slight modifications. The exploration of enzymatic activity involves pectate lyase (pectate transeliminase, E.C. 4.2.2.2), polygalacturonase (pectin depolymerase, pectinase, E.C. 3.2.1.15), amylases, lipases, proteases, phosphatase, DNase, RNase and urease.

(vi) Statistical analysis

All experiments were performed in triplicates with repetition. Statistical analysis was conducted to evaluate the significance of the study. Each value is mean of three parallel replicates. Standard error of the mean and Duncan's New Multiple range test (DMRT) [13] was used to indicate means with significant differences at $p \leq 0.05$.

RESULTS AND DISCUSSION

(i) Enumeration and identification of fungal isolates

Surface and sub-surface soils are very fertile precinct and are prosperous with an utmost diversity of soil inhabiting microorganisms. Soil dwelling microorganisms perform their life processes at this stratum. Normal garden soil and polluted soil were reported to be a preferred source for isolation of microbes, presumably because of the various biological activities that generate transient alkaline conditions in such environments[14]. Hence, soil samples were collected within 3-5 cm depth from different parts of Bhubaneswar and Keonjhar, Odisha, India. The technique adopted in this study for sampling is more appropriate to preclude the contamination of air spores[15]. Nevertheless, the contemporary method adopted to collect samples is highly unswerving and provides a precise number of microorganisms akin to other techniques reported[15]. Once the soil is removed from its innate hub, there ensues a change in various parameters which directly hamper soil microflora, particularly the anaerobes[16] which are unable to endure the changed conditions. Keeping this in view, samples were placed at 4 °C to preclude the loss of viability of fungal spores as low temperature is preferable for prolonged survival of spores[9, 15].

The Dilution plate technique was implemented for isolation of fungi. It is a very popular and efficient technique to get total counts of viable and culturable spores and hyphae[17]. Czapek's Dox agar medium was found to be greatly apposite. It is a recommended medium for isolation of fungi[18]. Utmost numbers with a variety of fungal community were recorded in the botanical garden soil of P.G. Department of Botany (3×10^8 CFU/g), Utkal University, Bhubaneswar, India followed by Mancheswar Industrial Estate (Table 1). Such results were obtained as the soil is prosperous in various organic matters due to the agro-horticultural practices, household and industrial organic pollutants owing to various activities. Other soil enumeration studies of different sites reported lower counts as compared to the botanical garden soil. At par counts were also documented by Fleet and Mian[19] in garden soil. Joda Industrial Estate and Barbil Industrial Estate of Keonjhar were also affluent in fungal biodiversity. Fungal identification was carried out by the aid of laboratory experiences, references of certain monographic books[10,11] and NCFT, New Delhi.

Table 1
Enumeration of fungal community (CFU/g) in soil samples of eastern and north-east regions of Odisha, India

Site for soil sample collection	Number of soil samples collected	Soil samples with maximum isolation	Percentage of maximum isolation	Total colony forming units (CFU/g)
BHUBANESWAR, ODISHA				
Patia Industrial Estate	35	23	65.7^{d}	5×10^{7}
Mancheswar Industrial Estate	35	27	77.1^{c}	1×10^{8}
Ganga Nagar	35	29	82.9^{b}	2×10^{7}
BDA Colony, CS Pur	35	26	74.3^{c}	2×10^{7}
Garden soil [a]	35	34	97.1^{a}	3×10^{8}
KEONJHAR, ODISHA				
Joda Industrial Estate	35	24	68.6^{d}	2×10^{7}
Barbil Industrial Estate	35	30	85.7^{b}	7×10^{7}

[a] Garden soil: botanical garden soil of Post Graduate Department of Botany, Utkal University, Bhubaneswar, Odisha, India.
Data pooled from a total of 3 separate experiments each comprising of 3 replicates.
(Mean values within column with different superscript alphabets are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test).

Soil enumeration study revealed that *Aspergillus niger* was most frequent and abundant species followed by *Aspergillus terreus* (Fig 2) in direct plate isolation whereas *Aspergillus candidus* was dominant in botanical garden soil followed by *A. niger* when isolated by dilution plate technique (Fig 3).

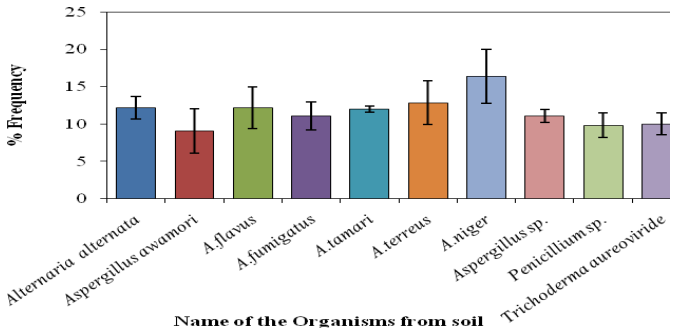

Figure 2
Fungi and their percentage frequency recorded from soil (Direct plate) (mean values ± SEM) of botanical garden, P.G. Department of Botany, Utkal University, Odisha, India

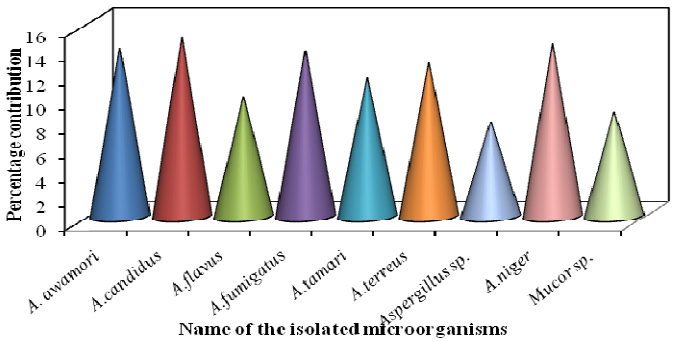

Figure 3
Percentage contribution of fungi recorded from soil (Dilution plate) of botanical garden, P.G. Department of Botany, Utkal University, Odisha, India

From Table 2, it is evident that a total of 1245 fungal strains were isolated. Garden soil is rich in various fungal communities representing 458 species followed by Ganga Nagar. There was much disparity in the occurrence of fungal community at these seven sites selected for the investigation. A total of 35 fungal taxa were isolated exhibiting 9 genera and the genus *Aspergillus* was most abundant and predominant one (Table 2; Fig 2 and 3). The genus *Alternaria, Curvularia* and *Fusarium* have represented two taxa, *Penicillium* by four taxa and *Trichoderma* represented only one species. *Mucor* sp. and *Rhizopus* sp. were having two and three isolates, respectively that belonged to the sub division Zygomycotina. Even after a short exposure to U.V. radiation, only one species was never sporulated in the agar medium implemented in this study and thus recorded as white sterile mycelium. The colony morphology of *Aspergillus terreus* is cinnamon to brown, furrowed and velvety with brown reverse plate colour. Microscopic study revealed that the conidiophores were 100-250 × 4.5-6 µm, vesicle: hemispherical, 10-15 µm, merging into supporting conidiophores, biseriate sterigmata, primaries crowed, parallel 5-7 × 2-2.5 µm, secondaries closely packed 5.5-7 × 1.5-2 µm, conidia: short, smooth, colourless, globose to slight elliptical, 1.5-2.5 µm in diameter. *Philides were* biseriate; vesicle: round, loosely radiate head. Hulle cells were solitary, round and produced directly on hyphae with colourless head (Fig 4). The identifications made based on macro and microscopic characteristics were not adequate enough so as to steer the identification up to the species level, but authenticates their association up to the genus level. A thorough specific identification is also obligatory, which is on the go. Fungi signify a cluster of microorganisms that are abundant in environment especially in soil[20]. The genus *Aspergillus* is the most bountiful fungus among all soil fungi[21], which is akin to the observation that we concluded during this study.

Figure 4 (**L-R**)
Mixed fungal culture isolated from garden soil samples, pure culture of Aspergillus terreus (quadrant streaking) and Phase Contrast Microscopic view of A. terreus

Table 2
Occurrence of fungal isolates in polluted soils of eastern and north-east regions of Odisha, India

Name of Fungus	Soil sample collection sites							
	Bhubaneswar					Keonjhar		
	Patia	Mancheswar	Ganga Nagar	BDA Colony	Garden soil [b]	Joda	Barbil	Total
Alternaria alternata	2	5	8	0	15	0	3	33
Alternaria tenuis	3	0	0	6	11	0	1	21
Aspergillus awamori	0	3	0	1	21	1	3	29
Aspergillus brevipes	4	0	3	3	7	2	0	19
Aspergillus candidus	1	1	5	3	8	4	5	27
Aspergillus clavatus	0	7	8	3	14	4	1	37
Aspergillus flavus	5	8	6	9	13	9	3	53
Aspergillus fumigatus	3	9	5	4	3	5	1	30
Aspergillus japonicus	0	0	0	3	8	4	1	16
Aspergillus kanagawaensis	0	4	6	1	11	0	0	22
Aspergillus niger	21	20	22	37	49	13	19	181
Aspergillus nidulans	2	0	9	4	17	7	13	52
Aspergillus parasiticus	0	3	0	0	3	1	0	7
Aspergillus oryzae	0	0	21	7	5	0	0	33
Aspergillus stellatus	0	3	11	1	33	0	5	53
Aspergillus sydowii	4	13	0	9	11	7	2	46
Aspergillus tamari	0	2	6	11	23	3	0	45
Aspergillus terreus	9	4	24	2	39	6	0	84
Aspergillus versicolor	0	0	4	0	10	0	0	14
Aspergillus species	1	1	11	17	16	0	7	53
Curvularia lunata	2	0	0	0	18	0	0	20
Curvularia pallescens	6	15	0	0	20	0	6	47
Fusarium oxysporum	7	0	9	0	0	0	0	16
Fusarium sp.	3	15	11	0	18	13	0	60
Mucor hiemalis	1	0	0	11	22	1	0	35
Mucor sp.	1	1	9	8	14	0	0	33
Penicillium sp.1	0	0	3	0	7	0	0	10
Penicillium sp.2	4	0	17	0	0	3	2	26
Penicillium sp.3	14	0	0	0	3	1	0	18
Penicillium sp.4	0	3	1	11	4	2	1	22
Rhizopus nigricans	9	0	6	0	15	0	0	30
Rhizopus sp.1	0	3	1	7	4	1	2	18
Rhizopus sp.2	6	0	3	0	10	0	0	19
Trichoderma aureoviride	1	1	5	0	6	1	3	17
White sterile mycelium [c]	2	11	0	2	0	0	4	19
Total	**111**	**132**	**214**	**160**	**458**	**88**	**82**	**1245**

[b] Garden soil represents the botanical garden soil of Post Graduate Department of Botany, Utkal University, Bhubaneswar, Odisha, India. [c] The species was not sporulated in the agar medium even after a short treatment with UV radiation and was noted as white sterile mycelium.

(ii) Hydrolytic enzyme diversity

An arrayof hydrolase producers that exist in nature as saprophytes[22] have been isolated by various isolation methods including soil dilution and soil plate methods. Myco-hydrolytic enzymes contribute 40 % of worldwide enzyme market and have numerous industrial exploitations[23]. Therefore, there is an incessant exercise going on in research on screening of these aboriginal fungal strains having enhanced enzyme biosynthesizing capabilities. Keeping in view of the credential, 35 local fungal isolates isolated from environmental sources were subjected to screening for the ability to biosynthesize extracellular hydrolases which occupy their pivotal place in diverse fields i.e. from fermentation industries to diagnostic laboratories. Most of the isolates biosynthesized one or more hydrolytic enzymes investigated in this study. Only two strains (1 strain of white sterile mycelium and 1 strains of *Fusarium* sp.) were not produced any of the hydrolytic enzymes evaluated. Six fungal isolates biosynthesized all the investigated enzymes and *Aspergillus terreus* was best among them (Table 3; Fig. 7). Six different

industrially important hydrolases produced by *Penicillium* and *Trichoderma* species has also been documented earlier[24]. In this course of investigation >50 % of isolates were found pectinolytic, among them 4 strains were good producers (Table 3). The genus *Aspergillus* is regarded as the key pectinases biosynthesizers[25].

Thirty three strains out of thirty five were found amylolytic and 17 were recorded as good producers of amylase as evaluated on the basis of size of halo around the colonies in starch agar plates when stained with iodine solution. Most of the amylolytic strains are from the genus *Aspergillus,* the prevailing amylolytic genera in nature[26]. A total of twenty-two isolates were lipase positive strains (Table 3). Several microorganisms, such as *Aspergillus terreus*[27], *Mucor hiemalis f hiemalis*[28], *Penicillium citrinum*[29], *Fusarium solani*[30], *Yarrowia lipolytica*[31] and *Trichoderma* sp.[32] are also exploited for lipase biosynthesis. The present investigation on the diversity of hydrolases divulges that protease is the most widely biosynthesized enzyme in all isolates followed by amylase and lipase as given in Table 6. Earlier findings also corroborates the biosynthesis of high levels of proteases by soil dwelling strain of *Mucor hiemalis*, *Mucor racemosus* and *Mucor bacilliformis*, *Mucor miehei*[33], *Rhizopus* species[34] and *Penicillum* sp.[35]. A variety of *Aspergillus* species owing to their noteworthy biosynthesis such as *A. oryzae*[36], *A. terreus*[37], *A. niger*[38], *A. clavatus*[39], *A. awamori*[40] and *A. fischeri*[41] were reported to secrete extracellular proteases and other enzymes. Besides to that thirteen strains were found to produce phosphatase, sixteen species for DNase, twelve strains for RNase and seventeen for urease were represented in Table 3.

Figure 5 (**L-R**)
Screening of Aspergillus terreus for its hydrolytic potentials (pectin depolymerase, pectate transeliminase, Amylase, lipase, protease, phosphatase, DNase, RNase and urease)

Table 3
Hydrolytic potential of fungal isolates isolated from eastern and north-east region of Odisha, India

Name of the Fungus [a]	Types of hydrolytic enzymes studied [b]								
	Pectate transeliminase	Pectin depolymerase	Amylase	Lipase	protease	Phosphatase	DNase	RNase	Urease
Alternaria alternata	-	+	+	-	++	-	-	-	-
Alternaria tenuis	+	-	+	+	+	-	-	-	-
Aspergillus awamori	-	+	++	+	++	-	+	-	+
Aspergillus brevipes	+	-	+	-	+	-	-	-	-
Aspergillus candidus	-	-	+	-	+	+	+	+	+
Aspergillus clavatus	-	+	+	+	++	+	-	-	-
Aspergillus flavus	+	+	+	+	+	+	+	+	+
Aspergillus fumigatus	-	+	+	+	+	-	-	-	+
Aspergillus japonicus	-	-	-	-	-	-	+		
Aspergillus kanagawaensis	-	+	+	+	+	-	+	+	+
Aspergillus niger	++	++	++	++	++	++	+	+	+
Aspergillus nidulans	+	-	+	+	+	+	+	-	-
Aspergillus parasiticus	-	-	+	-	+	-	-	-	+
Aspergillus oryzae	+	+	++	+	+	-	-	-	-
Aspergillus stellatus	-	+	++	-	++	-	-	ND	ND
Aspergillus sydowii	-	+	+	+	+++	-	+	-	-
Aspergillus tamarii	-	+	+++	++	+	+	+	-	-
Aspergillus terreus	+++	+++	+++	+++	+++	++	+++	+++	+
Aspergillus versicolor	-	-	+	-	+	+	-	-	-
Aspergillus species	+	+	++	+	++	-	+	+	+
Curvularia lunata	+	-	+	-	+	-	-	-	-
Curvularia pallescens	+	++	+	+	++	-	+	-	-
Fusarium oxysporum	+	ND	+	-	+	-	-	-	+
Fusarium sp.	-	-	-	++	-	-	-	-	-
Mucor hiemalis	+	-	+++	+	+	+	+	+	+
Mucor sp.	+	+	++	+	+	-	-	-	+
Penicillium sp.1	++	++	+++	+	++	+	+	+	-
Penicillium sp.2	+	+	++	++	++	+	+	+	+
Penicillium sp.3	++	+	+++	++	++	-	-	-	-
Penicillium sp.4	-	+	++	+	+++	-	-	-	+
Rhizopus nigricans	+	+	+++	+	+++	+	-	+	+
Rhizopus sp.1	+	+	++	+	++	-	-	+	+
Rhizopus sp.2	+	-	+	-	++	_	-	ND	ND
Trichoderma aureoviride	+	+	++	-	++	+	+	+	+
White sterile mycelium [c]	ND	ND	-	-	-	ND	-	-	-

-: absent; +: less to moderate; ++: good; +++: exuberant; ND: not detected. [a] Incubation of plates for enzymatic hydrolysis was performed at 30 °C. [b] Hydrolytic potentials of the fungal isolates were evaluated using the specified medium described by Hankin and Anagnostakis[12] with slight modifications.

CONCLUSION

In this study, it is concluded that the aspergilli were admirable over producers of many enzymes. They are also evaluated as the excellent biosynthesizers of some heterologous proteins[7]. Hence, this investigation may light the path towards the genetic manipulation of the isolates for strain improvement. They can be manipulated/implemented for competent and absolute degradation of plant-based biomass and various industrial wastes that may help in solving pollution tribulations, which otherwise might be caused by their dumping. Exploiting such novel intrinsic fungal isolates, high valued-low cost metabolites can be biosynthesized from economic substrates (waste and effluents) addressing the welfare of humankind.

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