Article

Diesel degradation by residual substrate of *Agaricus bisporus* at the microcosm level

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Abstract

In Mexico there are large areas of soils contaminated by hydrocarbons, causing economic and social damage to agricultural production, in this sense, the need to seek economic alternatives that allow contributing to the recovery of affected agricultural soils arises. The present work aimed to determine the biodegradation of diesel in an agricultural soil using residual substrates (RS) of *Agaricus bisporus*. Soil contaminated with 7 039 ppm of diesel was used with different doses of RS, incubated for 28 days at 37 ºC. CO_2 production, diesel biodegradation, initial and final population of fungi, as well as specific enzymatic activity of initial and final laccases were determined. In all treatments, removal increased significantly (p= 0.001) at 37 °C, as well as CO_2 production rates. Treatment T4 had the highest percentage of diesel biodegradation (68.747%) and a final cumulative production of 6.144 x 10^{-4} mmol CO_2 m^{-3}. The activity of laccases and tolerant fungal populations decreased in all treatments; in addition, the bacteria increased from 7.6 to 8.9 log CFU g_{ss}^{-1}. Therefore, the diesel biodegradation activity is attributed to bioaugmentation and biostimulation by the residual substrate of *A. bisporus*.

Keywords: bioaugmentation, biodegradation, biostimulation, enzymatic activity.

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Introduction

In Mexico, Agriculture is one of the main primary activities that contributes 2.3% of the gross domestic product (GDP) (INEGI, 2020). The municipality of Acatzingo, Puebla-Mexico, is one of the main agricultural producers of crops such as: corn, beans, alfalfa, cabbage, lettuce, nopal, tomato, prickly pear and carrot mainly (SIAP, 2021). However, these agricultural areas have been affected by the intensive use of chemical fertilizers and pesticides, as well as by spills of diesel, gasoline or crude oil (Cavazos-Arroyo *et al*., 2014). Environmental deterioration with respect to soil degradation generates a cost of 0.41% of GDP (INEGI, 2018). Diesel is a pollutant that causes health problems due to the presence of polyaromatic hydrocarbon (PAH) compounds, which are considered genotoxic, mutagenic and carcinogenic (Schulte and Hauser, 2012). For all this, the need to recover agricultural soils contaminated with diesel in this region arises, because it is the main source of subsistence for peasants.

The 'mushroom' *Agaricus bisporus* contributes 15% ($34x10^6$ t) of the world's production of edible fungi and is the fourth most cultivated species, mainly for its flavor and nutritional, functional and medicinal properties (Royse *et al*., 2017). In Mexico, the production of *A. bisporus* was 59 349 tonnes, representing 93.7% of the national production of fungi, where it maintains the leadership in Latin America (Martínez-Carrera *et al*., 2016). During the growth and fruiting of *A. bisporus*, approximately 44% cellulose, 29% xylan and 8% lignin are degraded; however, there are 20 to 30% of the polysaccharides present in the substrate that remain undegraded; this calculation does not consider the conversion of substrate into vegetative mycelium (Kapu *et al*., 2012; Vos *et al*., 2017), thus, a significant part of the substrate can be used as a residual or post-harvest substrate (RS).

On average, a producing plant generates 5 kg of residual substrates (Lau *et al*., 2003) for each kg of fungi harvested, obtaining up to 24 t of residual substrate per month (Singh *et al*., 2011). The residual substrate has been studied as animal feed (Kim *et al*., 2011; Li *et al*., 2018), for the purification and extraction of enzymes, for applications of production of biogas, biofuel or bioremediation (Phan and Sabaratnam, 2012; Wan and Li, 2012), as it presents high concentrations of nutrients, microorganisms and enzymes (Ball and Jackson, 1995; Chiu *et al*., 1998; González-Matute *et al*., 2011; Kapu *et al*., 2012). Therefore, this research aimed to evaluate the residual substrate of *A. bisporus* in the bioremediation of an agricultural soil contaminated with diesel at the microcosm level, allowing to offer a biotechnological alternative for the restoration of agricultural soils impacted with hydrocarbons.

Materials and methods

A sampling of uncontaminated agricultural soil was carried out in Acatzingo, Puebla, Mexico with the following geographical coordinates 18º 57' 01" north latitude and 97º 43' 40" west longitude. The residual substrate of *Agaricus bisporus* used in this study was obtained from local producers from the city of Puebla, Mexico.

Physical, chemical and microbiological characterization of the soil and residual substrate of *A. bisporus*

The physical and chemical characterization of the agricultural soil and the residual substrate of *A. bisporus* was carried out as described in NOM-021-SEMARNAT-2000 (DOF, 2002). For the microbiological characterization of the soil and the RS, serial dilutions and the plate-counting technique were performed in differential culture media for the quantification of populations of bacteria, fungi, actinomycetes and bacteria tolerant to diesel expressed as $\log_{10}$ CFU g_{ss}^{-1} (colony-forming units/gram of dry soil).

The mesophilic bacterial population was quantified in nutritive agar medium (Bioxon, Mexico), the fungi in the potato dextrose agar (PDA) medium, the actinomycetes in Czapek Dox Agar and the diesel-tolerant bacteria in the basal medium; the latter with the following preparation (g L^{-1}): NH_4NO_3, 1; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_47H_2O$, 0.409; $CaCl_2$, 0.02; $FeCl_3$, 0.00005; bacteriological agar, 6.5; pH 7, supplemented with 100 µl of sterile diesel distributed on the gelled agar (Mauricio-Gutiérrez *et al*., 2014). Mesophilic bacteria, tolerant bacteria and actinomycetes were incubated at 30 °C for 72 h, fungi were incubated at 25 °C for 72 h.

Diesel biodegradation at microcosm level using RS of *A. bisporus*

The soil used was sterilized and intentionally contaminated with 7 039 ppm of diesel and weathered for three months. A completely randomized experimental design was used in a microcosm system to evaluate the participation of microbial populations and laccase (Lac) activity present in the study system with four treatments with different soil: substrate ratios on a dry basis (T1 (95: 5), T2 (90: 10), T3 (85: 15) and T4 (80:20)) and the contaminated soil ($T0_1$) and RS ($T0_2$) were used as controls.

For each treatment, serological bottles of 120 ml were used, with 30 g of the soil-substrate mixture and were adjusted to the C: N: P ratio to 100: 10: 1 using sterile solutions of NH_4SO_4 1N and K_2HPO_4 1N. Moisture was maintained between 23.8-25.6% ±5.23. The atmosphere of the bottles was exchanged every third day with an airflow (1.8 ml air/sec); through a sterile membrane (0.22 µm), the amount of CO_2 produced was previously evaluated. All treatments were incubated for 28 days at two temperatures (25 and 37 ºC).

The percentage of diesel biodegradation (%), actual density by means of the NOM-021-SEMARNAT-2000, initial and final activity of laccases (Lac), initial and final population of diesel-tolerant bacteria and fungi (DOF, 2002) were determined. All treatments were carried out in triplicate. The percentage of biodegradation was determined according to the following equation: percentage of biodegradation= $[(A - B)/A]*100$, A= initial concentration of diesel; B= final concentration of diesel

Basal respiration

The CO_2 produced by the soil for 24 h was quantified and absorbed by 5 ml of NaOH 0.1 N, calculated through titration with HCl 0.1 N using phenolphthalein, the excess of carbonates was previously precipitated with 5 ml of $BaCl_2$ 0.5 N (Rivera-Espinoza and Dendooven, 2004).

Diesel quantification

The initial and residual diesel was quantified based on the EPA Method 8015 C (Nonhalogenated Organics by Gas Chromatography) using a GC-MS with an HP-5 capillary column (30 m x 0.25 mm x 0.25 μm), whose initial flow was 1 ml min^{-1}. The conditions of analysis of the furnace were: initial temperature 50 ºC, maximum temperature 300 ºC.

Enzyme quantification

An extraction was carried out at the beginning and end of the experiment of the sample of the RS of *A. bisporus* and of the different treatments in soil: substrate ratios (5 g) with 20 ml of sodium acetate buffer (50 mM, pH 5) for 2 h at 80 rev min^{-1} and at 4 °C, then filtered through Whatman No. 1 paper. The extracts were stored at 4 °C for the determination of Laccases (Isikhuemhen and Mikiashvilli, 2009). The activity was determined by oxidation of 2.2'-azinobis, 3-ethylbenzothiazoline-6-sulphonate (ABTS) at 25 °C and read at 420 nm (ε_{420}= 36 000 mM^{-1} cm^{-1}), enzymatic activity was reported as specific activity (U g $soil^{-1}$) (Bollag and Leonowicz, 1984; Gayosso-Canales *et al*., 2011).

Microbiological analysis

The initial and final population of diesel-tolerant bacteria and fungi was quantified by direct plate counting. The determination of bacteria was performed for treatments incubated at 37 °C grown in basal medium mentioned above. The determination of fungi was for treatments incubated at 25 °C and the basal medium with the following chemical composition (g L^{-1}) was used: $(NH_4)_2SO_4$, 7; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_4 7H_2O$, 0.409; dextrose, 0.1; Sol. E 100x, 100 μl; rose bengal, 0.05; streptomycin sulfate, 0.05; pH 5.5. Diesel previously sterilized by filtration was used as a source of carbon, adding 100 μl on the petri dish (Mauricio-Gutiérrez *et al*., 2014). The microorganisms were reported as $\log_{10}$ CFU g_{ss}^{-1} (colony-forming units/gram of dry soil).

Statistical analysis

An analysis of variance to compare the different treatments and a Tukey multiple comparison test of means ($p< 0.05$) were performed using the Minitab Version 17.3 statistical package.

Results and discussion

The residual substrate (RS) derived from the edible fungus industry represents an environmental problem (Phan and Sabaratnam, 2012), in Europe 3.5 x 10^6 t are generated per year (García-Delgado *et al*., 2013). Mexico contributes to the generation of this waste, since it is the largest producer of edible fungi in Latin America, with *A. bisporus* being one of the main cultivated fungi (Martínez-Carrera *et al*., 2016). For these reasons, RSs have been studied for different applications such as bioremediation of soils contaminated with PAH (Cerniglia and Sutherland, 2010; Stabnikova *et al*., 2010; Pardo-Giménez *et al*., 2010; Upadhyay and Singh, 2011; Phan and Sabaratnam, 2012; García-Delgado *et al*., 2013).

Physical, chemical and microbiological characterization of soil and RS

According to the physical and chemical characteristics (Table 1) of the agricultural soil of Acatzingo, Puebla, Mexico, it was classified as loam-sandy, with low values of moisture (17.35%), organic matter (2.04%), total nitrogen (0.032%) and phosphorus (0.003%) compared to the parameters of the RS (190.32%, 57.62%, 1.46% and 0.79% respectively). In addition, it presented a moderately alkaline pH value (pH= 8.01). The RSs of *Agaricus bisporus* are characterized by high concentrations of nutrients, these properties have been used as biostimulant agents for the bioremediation of xenobiotic since they have high doses of nitrogen (2.16%), phosphorus (0.69%) and carbon (54.3%) (Corral-Bobadilla *et al*., 2019).

Table 1. **Physical, chemical and microbiological characterization of soil and RS of *A. bisporus.***

Parameters	Soil	Residual substrate
pH	8.01	7.26
EC (S cm^{-1})	205	Nd
Texture (%) [clay, sil, sand]	17.4, 18, 64.6	Nd
Moisture (%)	17.355	190.328
Total organic matter (%)	2.04	57.625
Total nitrogen (%)	0.032	1.46
Available phosphorus (%)	0.003	0.791
Actual density (g ml^{-1})	2.464	Nd
Bacteria (log CFU g_{ss}^{-1})	6.021	7.708
Fungi (log CFU g_{ss}^{-1})	3.455	5.554
Actinomycetes (log CFU g_{ss}^{-1})	2.895	3.559
Hydrocarbonoclastic bacteria (log CFU g_{ss}^{-1})	5.037	3.828

Nd= not determined; g_{ss}= grams of dry soil.

With respect to the quantification of cultivable microorganisms in Table 1, the bacterial population had the highest values in the soil (6.021 log CFU g_{ss}^{-1}) and the RS (7.708 log CFU g_{ss}^{-1}). The size of the other microbial populations varied between the samples, with the agricultural soil where there was a greater number of hydrocarbonoclastic bacteria (5.037 log CFU g_{ss}^{-1}), followed by the fungal population (3.455 log CFU g_{ss}^{-1}) and finally actinomycetes (2.895 log CFU g_{ss}^{-1}). However, in the RS there was a greater number of fungi (5.554 log CFU g_{ss}^{-1}) and the groups of actinomycetes and hydrocarbonoclastic bacteria remained at the same order of magnitude (3.559 and 3.828 log CFU g_{ss}^{-1}). The soil and RS harbor a native microbial population, the latter have been used as sources of bioaugmentation for bioremediation processes (Wang *et al*., 2016; Leong *et al*., 2022).

Diesel biodegradation at microcosm level using RS of *A. bisporus*

Diesel biodegradation experiments at the microcosm level (Table 2) showed a higher biodegradation at 37 ºC; treatment T4 was the one that obtained the highest removal (68.747%) followed by T4 at 25 ºC (61.261%) and T3 at 37 ºC (60.14%). On the contrary, the treatments with

the lowest percentage of removal were $T0_1$ (contaminated soil) at 25 ºC with a removal of 20.603%, followed by T1 (27.034%) and T2 (29.791%) at 25 ºC, presenting highly significant statistical differences (p= 0.001).

Table 2. Diesel biodegradation and production of cumulative CO_2 using RS of *A. bisporus.*

Treatment	Biodegradation		mmol CO_2 m^{-3} day*	
	25 °C	37 °C	25 °C	37 °C
	(%)		($x10^{-4}$)	
T1	27.034 c	40.966 bc	4.319 ±0.287 c	5.608 ±0.533 b
T2	29.791 c	48.408 b	4.343 ±0.266 c	5.685 ±0.564 b
T3	37.177 b	60.14 a	4.808 ±0.574 b	6.014 ±0.214 a
T4	61.261 a	68.747 a	5.785 ±0.46 a	6.144 ±0.429 a
T01	20.603 d	37.357 c	4.902 ±0.334 b	5.432 ±0.346 b
T02	Nd	Nd	6.173 ±0.245 a	5.767 ±0 b

*= ±standard deviation; Nd= not detected. Different letters in the column indicate statistically significant differences according to the Tukey test (p= 0.05).

The cumulative and quantified CO_2 generation in the study system indicated that treatments incubated at 37 ºC produced a higher concentration (mmol CO_2 m^{-3} day) (Table 2). The treatments that obtained the highest CO_2 production were $T0_2$ (RS) (6.173 x 10^{-4} mmol CO_2 m^{-3} day) at 25 ºC, T4 (6.144 x 10^{-4} mmol CO_2 m^{-3} day) and T3 (6.014 x 10^{-4} mmol CO_2 m^{-3} day) at 37 ºC. And the treatments: T1 (4.319 x 10^{-4} mmol CO_2 m^{-3} day) at 25 ºC, T2 (4.343 x 10^{-4} mmol CO_2 m^{-3} day), T3 (4.808 x 10^{-4} mmol CO_2 m^{-3} day) and $T0_1$ (contaminated soil) (4.902 x 10^{-4} mmol CO_2 m^{-3} day) at 25 ºC presented a lower CO_2 production rate. Margesin *et al*. (2007) positively correlated hydrocarbon degradation with microbial activity and biomass, results similar to those obtained in the present investigation.

A 4:1 ratio of soil: substrate (T4) biodegraded 68.747% of diesel in 28 days, which allows treating 6.92 t m^{-3} of soil, as mentioned by Sasek *et al*. (2003), who added RS of *A. bisporus* to a 4:1 ratio (soil: substrate) and obtained a degradation of 68.8% of PAH (polyaromatic hydrocarbons) at the end of 154 days in 2.8 m^3 of soil. While Mohammadi-Sichani *et al*. (2019) reported a degradation of 71.5% in three months of TPH (total petroleum hydrocarbons) in soil, applying 10% of RS of *A. bisporus*. In addition, the actual density of each treatment was determined in order to assess the RS of *A. bisporus* as a texturing agent for application in bioremediation of soils contaminated with hydrocarbons.

The results obtained indicated that there is no statistically significant difference in the actual density between the treatments, where the values are in the range of 2.36 to 2.536 g mL^{-1} (Table 3). The percentages of biodegradation obtained can be due to the physical and chemical properties that RS provides to the soil such as: texture, water retention capacity, porosity and contribution of essential nutrients such as nitrogen and phosphorus (Table 1), which allow stimulating degradative microbial activity, helping in soil bioremediation (Stabnikova *et al*., 2010; García-Delgado *et al*., 2013).

Table 3. Actual density and amount of contaminated soil treated by RS of *A. bisporus* applied.

Treatment	Actual density*	Treated soil/residual substrate
	(g ml^{-1})	(t m^{-1})
T1	2.536 a	32.85
T2	2.413 a	15.56
T3	2.43 a	9.76
T4	2.36 a	6.92
T01	2.334 a	Na
T02	1.729 b	Na

Different letters in the column indicate statistically significant differences according to the Tukey test (p= 0.05). Na= not applicable.

The population dynamics of diesel-tolerant bacteria in the study system indicated a highly significant statistical increase (p= 0.001) at the end of the experiment for all treatments: T1 (7.83 log CFU g_{ss}^{-1}), T2 (8.9 log CFU g_{ss}^{-1}), T3 (7.7 log CFU g_{ss-1}) and T4 (7.6 log CFU g_{ss}^{-1}). However, the population count of tolerant fungi showed a highly significant statistical decrease (p= 0.001) with population sizes of 4.8 to 5.6 log CFU g_{ss}^{-1} (Figure 1).

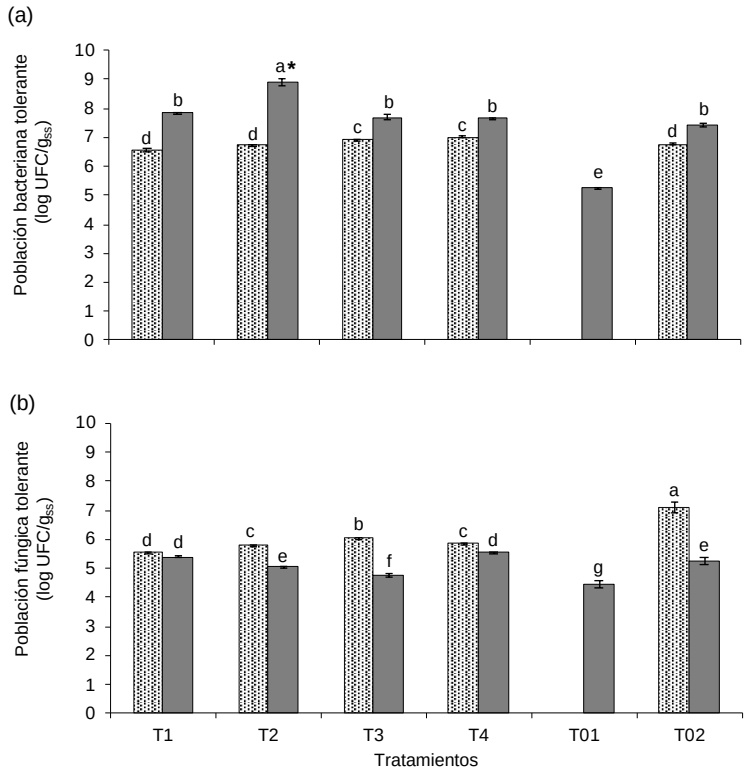

Figure 1. a) dynamics of the bacterial population of the treatments incubated at 37 °C; and b) of the fungal population of the treatments incubated at 25 °C tolerant to diesel. At the beginning (light bars) and end (dark bars) of the experiment with different soil: RS of *A. bisporus* ratios on a dry basis. Each value represents the average of triplicates. Different letters indicate statistically significant differences between treatments (p= 0.05) according to the Tukey test.

The quantification of Lac decreased significantly (p= 0) in the treatments incubated at 25 °C, quantifying specific enzymatic activities from 0.083 to 0.48 U g^{-1} (Figure 2). Treatment T4 (80:20) had the highest enzymatic activity (0.48 U g^{-1}) unlike the control group $T0_2$ (RS) of *A. bisporus* (2.46 U g^{-1}). In the treatments incubated at 37 °C, Lac activity was not detected according to the established methodology.

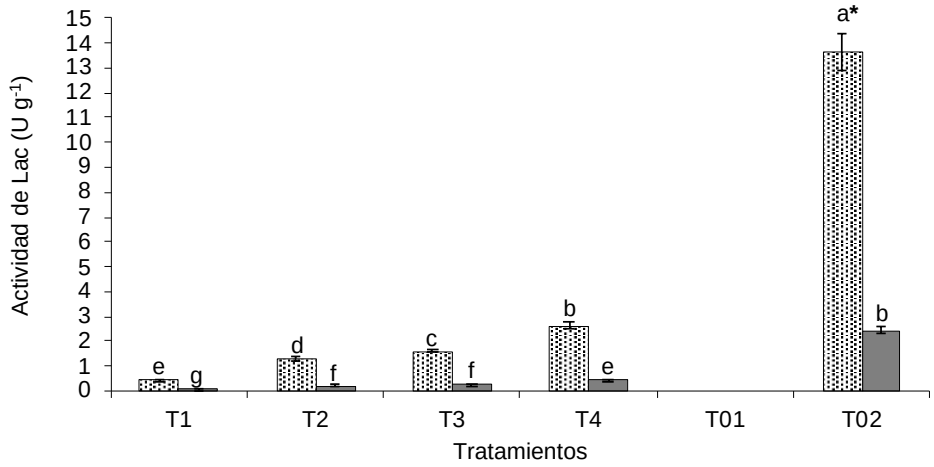

Figure 2. Laccases activity at the microcosm level with different soil: RS of *A. bisporus* ratios on a dry basis. Each value represents the average of triplicates of the treatments at the beginning (light bars) and end (dark bars) of the experiment. *= different letters indicate statistically significant differences between treatments (p= 0.05) according to the Tukey test.

The group of microorganisms studied in the biodegradation of diesel were the tolerant bacteria native to the RS and the soil, since they presented an effectiveness in the biodegradation and contributed to the bioremediation process (Gallego *et al*., 2001). On the contrary, treatments incubated at 25 ºC showed a decrease in the population of fungi since they were affected by the conditions of the microcosm (environment) and the type of contaminant used, as well as in the production of enzymes such as Lac. Bento *et al*. (2005) mention that microorganisms in a hostile environment decrease their metabolic activity and limit the growth of their population.

Although there are different reports of extracellular enzymes secreted by *A. bisporus* in RS that can be used in bioremediation processes (García-Delgado *et al*., 2013; Chatterjee *et al*., 2017). It is important to mention that the control treatment $T0_1$ (soil) presented a diesel biodegradation of 37.357%, this value may be associated with the loss due to volatilization since it can be 35-40% in soil (Rhykerd *et al*., 1999; Saviozzi *et al*., 2009) and the presence of reproductive structures of the native microbiota that resisted soil sterilization (Sylvia *et al*., 1999), since in the present study, $T0_1$ (soil) presented populations of bacteria and fungi of 5.3 and 4.4 log CFU g_{ss}^{-1}.

The RS of *A. bisporus* was characterized as a biostimulant agent for presenting organic nutrients, it also increased the porosity of the contaminated soil, improving oxygen diffusion and water retention capacity. It also presented bioaugmentation characteristics since it provided microbial load as a degrading source of diesel with a potential effect on the biodegradation of hydrocarbons.

As established by Chiu *et al*. (1998), where they mention that RSs have the advantage of degrading xenobiotic compounds by the consortium of enzymes of various microorganisms, in addition to being relatively rich in nutrients that stimulate these microorganisms in the secretion of enzymes without having any nutritional limitation.

In this context, there are several studies where different RSs are evaluated, such as: activated sludge, sugarcane bagasse, sugarcane filter cake and corn residues for purposes of bioremediation of soils contaminated with diesel with biodegradation percentages of 61 to 90% in a time of 15 to 109 days, results similar to those obtained in the present study (Gallego *et al*., 2001; Molina-Barahona *et al*., 2004; Rivera-Espinoza and Dendooven, 2004; Bento *et al*., 2005; García-Torres *et al*., 2011).

Conclusions

This research identified an alternative use the RS of *A. bisporus* for the bioremediation of agricultural soils contaminated with diesel, attributing this biodegradation to the native bacterial activity of RS and soil; in addition, the RS was an important source of nutrients. Therefore, further studies on the microorganisms and biodegradation routes involved are required, for small-scale and industrial application under real conditions in field trials.

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