INVITRO PRODUCTION & OPTIMITION OF PHB PRODUCING *BACILLUS CEREUS*

[1]J.ISHWARYA, [2]L jeeva

[1]Assistant professors, [2]Research scholar
PG and Research Department of Microbiology
Kamban college of Arts and Science for women
Thiruvannamalai,606603

***Abstract*- Plastics manufactured from biomass, such as corn, sugarcane, etc., are known as bioplastics. These chemicals are receiving more attention as ways to conserve fossil fuels, lower CO2 emissions, and reduce plastic waste. The biodegradability of bioplastics has received extensive public attention, and the demand for packaging among large-scale retailers and the food industry is rising quickly. Since plastic is not biodegradable, it is extremely unsafe to use it. Therefore, the usage of biodegradable plastics is becoming increasingly popular. All of these issues—applications, production, kinds, difficulties, sustainability, fermentation, process development, and utilization of inexpensive substrates—are included in the current review. With the usage of carbon sources, the bacterial isolate Bacillus cereus was observed to produce more PHB. However, when dextrose (0.4 g/l) was utilized as the only carbon source, production was decreased while maltose (6 g/l) increased production. The isolated PHB was mixed with thermoplastic starch in an experimental setting to maximize its potential usage. The product's crystalline nature was significantly enhanced after blending, as evidenced by the diffraction patterns at 2 values of 26.80°, 31.74°, 45.59°, and 56.22°. As a result, the bacterially mediated PHB synthesis can be used as a more effective alternative to address the present practices of plastic use and its continuously rising level of pollution.**

***Keywords*: acetoacetylcoA, acetyl, polyhydroxybutyrate**

INTRODUCTION

Almost every area of our life involves the use of plastics. Toys, furniture, and daily items like beverage containers were all made of plastic. Plastics are widely used, hence good end-of-life management is necessary. The majority of plastics were identified in packaging and containers, such as soft drink bottles, lids, and shampoo bottles, but they were also present in both durable and nondurable products, such as diapers, garbage bags, cups and utensils, and medical equipment. The extensive usage of plastics in modern culture is largely attributable to their advantageous thermal and mechanical characteristics, which make them a stable and long-lasting material. Due to improper disposal of plastics, the widespread usage of plastics worldwide had a significant negative impact on the environment.

Plastic bags had a reputation for harming the environment significantly. It can take a single plastic bag up to 1000 years to totally decompose. Due to their extended residence times (compared to degradable materials like paper), the bags accumulate far more on the natural landscape. In other words, the likelihood of environmental harm increases with the amount of plastic bags used.

More than 300 million tons of synthetic polymers (also known as "plastics") are produced annually and have largely replaced traditional materials like wood, stone, horn, ceramics, glass, leather, steel, concrete, and others. Applications that were not feasible prior to the availability of the materials were now enabled.

Bioplastics were made from renewable biomass sources such corn starch, vegetable fats, and oils. When compared to ordinary plastic, bioplastic can reduce carbon dioxide emissions by 30 to 70 percent. Both anaerobic and aerobic settings can facilitate the decomposition of biodegradable bioplastics.Our natural environment both directly and indirectly contributes to our survival and well-being. One of the main contaminants on planet was plastic. Bioplastic is being developed to replace synthetic plastic and to reduce pollution. The majority of the degraded organic waste used to create bioplastic came from microorganisms and algae.

Traditional single-use plastics can be replaced by biodegradable containers, bottles, and packaging manufactured of bioplastics. Bioplastics have a low environmental impact since they can decompose naturally over time and are made from renewable resources, such plants.

Poly (3-hydroxybutyrate, or PHB) is a thermoplastic polyester material that is totally biodegradable, renewable, and produced by bacteria. It has a crystalline fraction of over 80% and is highly hydrophobic. PHB's physical and chemical characteristics are somewhat reminiscent of several petroleum-based synthetic polymers.

As a result, PHB had been extensively researched as an eco-friendly polymeric material. PHB has a low extension at break and very poor mechanical qualities as a result of its high crystalline content, which restricts the variety of applications it can be used for. Due to PHB's greater cost in comparison to commercial polymers, high brittleness, and challenging processing, there has not previously been a significant commercial production of PHB products.

Gram-positive, rod-shaped, beta hemolytic Bacillus cereus was an endemic, soil-dwelling, gram-negative bacterium. While some strains can make people sick from eating them and cause food poisoning, other strains can make good probiotics for animals. The spore-forming bacterium Bacillus cereus can infect people and is found naturally in a variety of foods.

MATERIALS AND METHODOLOGY

The present investigation was to determine the production and optimation of PHB producing ***Bacillus Cereus*** from the dump soil.

OPTIMIZATION OF POLYHYDROXYBUTYRATE (PHB)

EXTRACTION OF PHB FROM CARBON SOURCES For three days at 37 C and 150 rpm in a rotary shaker, Bacillus cereus was grown in Minimal Davis Media supplemented with dextrose as a carbon source. PHB extraction was carried out using the sodium hypochlorite-chloroform method after three days of incubation. After centrifuging 20 ml of culture at 6,900 g for 20 min, the supernatant was separated.

The pellet was incubated at 37°C for 1 hour while suspended in 2.5 ml of hot chloroform and 2.5 ml of 4% sodium hypochlorite for digestion. The suspension was centrifuged at 1500 rpm for 10 minutes (the intermediate phase is chloroform containing cell debris, and the upper phase is hypochlorite solution). The bottom phase that included PHA and chloroform was collected, and then extraction with hot chloroform was performed. Finally, ethanol and acetone (1:1) were used to precipitate the mixture. To get PHA crystals, the precipitate was allowed to dry out at 30°C (Singh and Parmar, 2011).Other carbon sources, such as dextrose, lactose, sucrose, maltose, fructose, and galactose, were also used in the same way.

MORPHOLOGICAL CHARACTERIZATION TECHNIQUES

Gram staining , capsule staining, indole test, methyl red test, voges proskauer test, citrate utilization test, sugar test, catalase test , oxidase test.

SUDAN BLACK STAIN

Sundan black powder: 0.06g

Ethyl alcohol : 70ml

PROCEDURE: Prepare nutritional agar with 1% glucose. 24 hours were spent incubating the injected culture.Flood the colony with 0.02% Sundan Black B (ethanolic) and leave it alone for 30 minutes.96% ethanol was used as a rinse to get rid of extra discoloration from colonies. A black colony was seen.

SUDAN BLACK STAIN METHOD microscopic determination using the sudan black stain method.Bacillus cereus was cultured, and a thin smear was created on a sterile, clean glass slide.By repeatedly burning the slide over a burner, heat was able to remove the stain.Sudan black B solution was applied sparingly and left on the fixed smear for 5–10 minutes. The slide should be submerged in xylene until totally decolored. Let the slide dry out. Flooded the slide with counterstain, soaked it in the saffron solution for ten seconds, then gave it a gentle rinse under running water before letting it air dry. Examine the slide using an immersion lens filled with oil.

CARBOL FUCHSIN STAINING:

To ascertain the isolate's intracellular PHB production, carbol fuchsin staining was used. Carbol fuchsin stain was applied to a tiny smear of each isolated object for 45 seconds. The PHB-producing isolates displayed dark-colored PHB granules inside their cells.

Use of the Carbol fuchsin stain as reagents.

By taking culture, a thin smear was created on a spotless, sterile glass slide.By repeatedly burning the slide over a burner, heat was able to remove the stain.For 30 seconds, flood with 0.2% carbol fuchsin.Before performing a microscopic examination at a 1000 times magnification, wash the stain off and let it air dry or gently blot it dry.

Carbol fuchsin staining was performed to determine the intracellular production of PHB by the isolate. A thin smear of all the isolated were stained with carbol fuchsin stain for 45 s. The isolates capable of Producing PHB showed dark colored granules of PHB intracellularly.

Reagents used: Carbol fuchsin stain.

Thin smear was made in a clean sterile glass slide by taking culture.Heat fixed the smear by flaming the slide over a burner a few times.Flood with 0.2 % carbol fuchsin for 30 seconds.Wash off the stain and either air dry or gently blot dry before microscopic examination at 1000 times magnification.

OPTIMIZATION OF CULTURAL PARAMETERS FOR MAXIMUM PHB PRODUCATION:

The various elements influencing the chosen bacterial isolates' generation of PHB were optimized.

EFFECT OF DIFFERENT TEMPERATURES

The medium sucrose/yeast extract broth medium was made, and the pH level was adjusted to 7.0.The conical flask (250 ml) containing 100 ml of medium and the bacterial isolate were sterilized at 121 oC for 20 min. PHB was measured after 48 hours of incubation on a rotary shaker at 20, 30, and 40 oC and 150 rpm.

EFFECT OF DIFFERENT PH

The bacterial isolate was sterilized at 121°C for 20 minutes while growing in a conical flask (250 ml) containing 100 ml of medium. The inoculated flasks were incubated at 30°C for 48 hours at 150 rpm, and PHB was measured. The medium sucrose/yeast extract broth medium were made with varied pH ranging from (6, 7, 8 and 9) and the inoculated flasks.

EFFECT OF DIFFERENT INCUBATION TIMES

The bacterial isolate was cultured in a conical flask (250 ml) with 100 ml of a broth medium containing sucrose and yeast extract and adjusted to a pH of 7.0. The flasks were then sterilized at 121 oC for 20 minutes.The inoculated flasks were incubated

for 24, 48, 72, and 96 hours at 30 degrees Celsius and 150 revolutions per minute.Quantification of the PHB generated after 48 hours.

EFFECT OF DIFFERENT MEDIA

Different media, including synthetic and sucrose/yeast extract medium, were used. The bacteria were sterilized at 121°C for 20 minutes before being cultured in conical flasks (250 ml) containing 100 ml of each prior media. PHB was measured after 48 hours of incubation at 30 oC and 150 rpm in the inoculated flasks

EFFECT OF DIFFERENT CARBON

The chosen bacterial isolate was cultured in 250 ml conical flasks with 100 ml Sucrose yeast extract broth medium and various amounts of carbon sources, including glucose, sucrose, mannitol, lactose, whey, and molasses (1%, 2%, and 3% w/v). For 48 hours, the flasks were incubated at 300C while being rotated at 150 rpm. According to Miller Santimano et al. (2009), PHB generated by the isolates was measured after incubation.

EFFECT OF DIFFERENT NITROGEN SOURCES ON PHB PRODUCTION

The bacterial isolate were grown in 250 ml conical flasks containing 100 ml sucrose yeast extract broth medium with the best carbon source, and different N sources were used like ammonium sulfate, ammonium chloride, and yeast extract, all at different concentrations (0.5, 1, 1.5 g/L). After 48 hr., PHB yields were quantified.

THIN LAYER CHROMOTOGRAPHY:

TLC was used to qualitatively check for PHB in the extracted PHB granules. The mobile phase Benzene: Chloroform (2:1) separated the extracted PHB, and the Rf value was computed. TLC was performed on silica plates that had calcium carbonate coatings. After being spotted on the TLC plates, the material was left to air dry at room temperature. The mobile phase (1:1) of benzene and ethyl acetate was used to separate the sample, and the plate was left to do so.Iodine vapour was used to visualize the spots, and Rf values were computed.

RESULT AND DISCUSSION

Since the invention of the automobile, plastic has been a crucial component of our daily life. The goal of the current experiment was to cultivate and improve the PHB-producing Bacillus Cereus.

It was determined by that the bacterial isolate used in this study was Bacillus cereus. It responded favorably to Gram's response and had distinctive petriplate characteristics. When extracted with sodium hypochlorite, the PHB-producing strain displayed unusual features. Sodium hypochlorite persists in the top layer following cell lysis, followed by cell debris in the middle phase and PHB precipitating with addition of chloroform.

In this investigation, dextrose, sucrose, maltose, fructose, and galactose were used as carbon sources. The PHB were lyophilized after extraction, and dry weight measurements were made (Fig. 15). Out of the carbon sources tested, maltose presence resulted in a higher level of PHB production (0.563 g), but dextrose presence resulted in a lower level of production (0.04 g). TABLE(1) contains information about the quantity of PHB produced in detail

The PHB blend produced was found to be brittle and breaks easily. Blending PHB with other polymers is an economic way to improve its mechanical properties. To this effect PHB was blended with thermo stable starch. PHB and starch was not completely miscible and the blend showed insoluble particle aggregation on the surface . The maximum PHB production occurred at 30°C after 47hrs of incubation. The production of PHB increased were was recorded.

The created PHB blend was discovered to be easily breakable and fragile. PHB can have its mechanical qualities improved economically by blending with other polymers. In order to achieve this, thermally stable starch was mixed with PHB. PHB and starch weren't entirely miscible, and the mixture's surface exhibited insoluble particle agglomeration. After 47 hours of incubation, the greatest PHB production occurred at 30 °C. There was evidence of increased PHB production.

Thin layer chromatography was used to characterize the PHB, and its RF value is 0.4. Every year, more plastic garbage is generated, and it is unclear how long it will take for that debris to biodegrade. New biodegradable polymers have been developed as a result of environmental consciousness.

Plastics manufactured from biomass, such as corn and sugarcane, are known as bio-plastics. These chemicals are being emphasized more and more as ways to conserve fossil fuels, lower CO2 emissions, and reduce plastic waste. The bioradability of bioplastics has received extensive public attention, and the demand for packaging among large-scale retailers and the food industry is rising quickly.

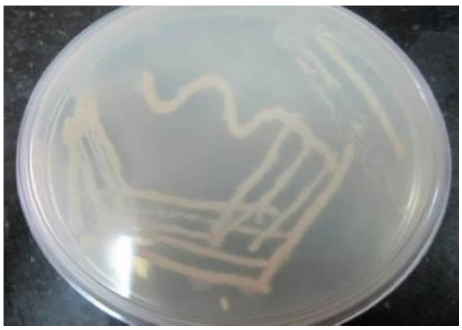
The increase in human population has caused an enormous volume of non-biodegradable trash to accumulate around the world. Environmentally speaking, the buildup of plastic garbage has become a big concern (Saharan and Badoni, 2007).

Conventional plastics produce poisons when they break down and take several years to completely decompose. Therefore, it is necessary to create plastics in a "eco-friendly" manner from components that can be easily removed from our biosphere (Gross and Kalra, 2002). Bioplastics are organic biopolymers that have been created and broken down by diverse species. Under stressful circumstances, they build up as storage materials in microbial cells. However, bioplastics were long overlooked due to their high production costs and the accessibility of less expensive petrochemical-derived polymers.

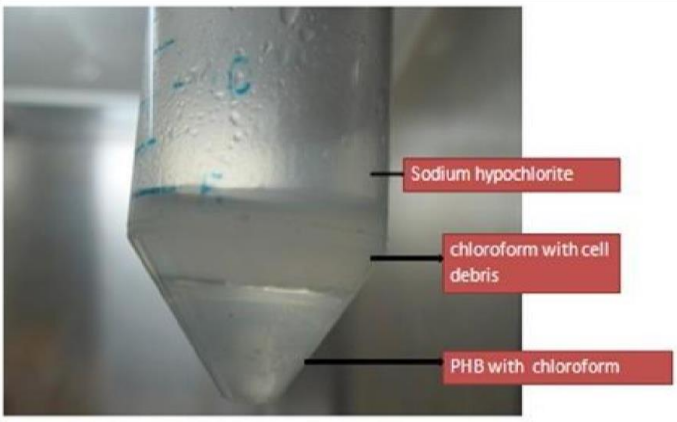
A homopolymer of 3-hydroxybutyrate with a high melting point and high levels of crystallinity is known as poly (3-hydroxybutyrate) (PHB). PHB is a thermoplastic and one of the poly hydroxyalkanoates (PHAs) family members that has been studied the most.

Chloroform and sodium hypochlorite solution dispersions were utilized in this experiment to recover microbiological PHB. Hypochlorite treatment alone resulted in such significant deterioration, and when hypochlorite concentration increased, so did the molecular weight. Different carbon sources had an impact on how much bacteria grew and how much PHB was extracted.

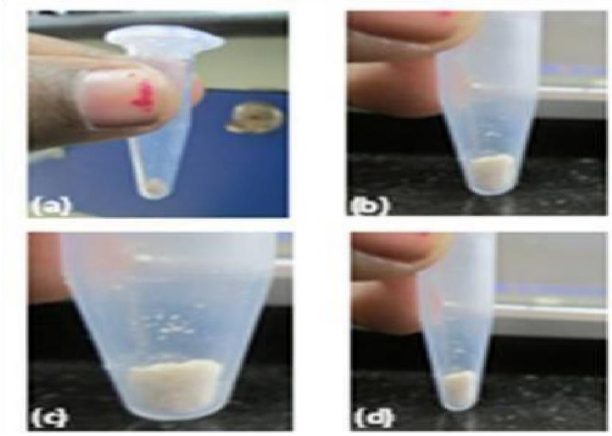
PHB is a sort of bioplastic that offers an excellent alternative to petrochemical plastic since it is easily biodegradable, biocompatible, and environmentally friendly. Bioplastics are a unique kind of biomaterial. Further research is required to determine whether the compatible and degradable nature of PHB as an alternative to petrochemicals is a viable option.



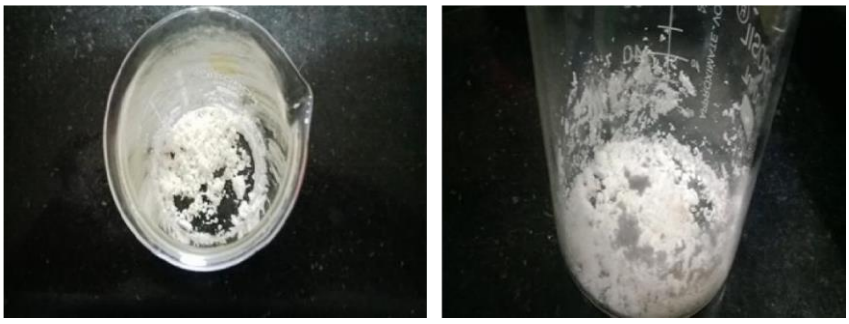
***BACILLUS CEREUS* SE-1 GROWN ON LURIA BERTANI AGAR PLATE**



THREE LAYERS OBTAINED DURING EXTRACTION OF PHB BY SODIUM HYPOCHLORITE METHOD



PRODUCTION OF PHB FROM DIFFERENT CARBON SOURCES LIKE (A) DEXTROSE (B) SUCROSE (C) MALTOSE (D) FRUCTOSE



THERMOPLASTIC STARCH BLEND WITH PHB

Table 1: OPTIMIZATION OF PHB FROM DIFFERENT CARBON

Carbohydrate source	**Cell biomass/ 2 ml (in g)**	**PHB production/ 100 ml (in g)**
Dextrose	0.024	0.04
Sucrose	0.026	0.281
Maltose	0.097	0.563
Fructose	0.079	0.361
Galactose	0.074	0.362

Table 2: FUNCTIONAL GROUPS IDENTIFIED BY FOURIER TRANSFORM-INFRARED SPECTROSCOPY ANALYSIS OF PHB

Wave number (cm-1)	**Functional groups**
1320–1000	C-O-C stretching of esters
1300–1150	-CH stretching
1470–1450	-CH_3 vibration
1730–1715	C=O stretch of ester
1680–1640	-CH_2 stretching
3500–3200	-OH stretching

TABLE 3: BIO-CHEMICAL CHARACTERIZATION OF THE ISOLATES

TEST	**RESULTS**
Gram staining	Positive
Capsule staining	Positive
Sudan black staining	Positive
carbolfuchsin staining	Negative
Indole	Negative
MR	Negative
VP	Positive
Citrate utilization	Positive
Catalase	Positive
Sugar	Negative
Oxidase	Negative

CONCLUSION;

Development of PHB as potential substitute material to some conventional plastics has drawn much attention due to the biodegradable and biocompatible properties of PHB. The potential applications of PHB in various industries and in the medical filed are encouraging.

Using conventional plastics comes with a multitude of drawbacks: the large amount of energy that is required to produce the plastic, the waste that is a result of plastic production, and the use of materials that do not biodegrade readily.In order to shift the production of plastics towards a more sustainable path, research is being conducted to

determine the types of renewable bioplastic resources that could be converted into plastic form PHB was extracted from different carbon sources (e.g. dextrose, lactose, fructose, maltose, galactose), where maltose produce more amount of PHB (0.562 g /100 ml culture). Characterization of extracted PHB was carried out by FT-IR, and XRD. Results showed that the bacterial culture accumulated about 7.75g of PHB was extracted from different carbon sources

REFERENCES:

1. Asada Y., miyake M., Miyake J., Kurane R., Tokiwa Y.,. Photosynthetic accumulation of poly –(hydroxybutyrate) by cyanobacteria- the metabolism and potential for CO2 recycling. 25(1-3):37-42
2. A. M. Gumel Y. Chisti, M. S. M. A. (2013) 'Recent Advances in the Production, Recovery and Applications of Polyhydroxyalkanoates', Journal of Polymers and the Environment, 21(2), pp. 580–605.
3. Ahn, W. S., Park, S. J. and Lee, S. Y. (2001) 'Production of poly(3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant Escherichia coli', Biotechnology Letters. Kluwer Academic Publishers, 23(3), pp. 235–240.
4. Aarthi.N, Ramana.K.V(2011). Identification and Characterization of Polyhydroxybutyrate producting bacillus *cereus* strains. *International journal of environmental sciences. 1(5);744-756*
5. About Bioplastics,http://www.sustainableplastics.org/about,accessed 2015.
6. Ashby, R. D. and Foglia, T. A. (1998). 'Poly(hydroxyalkanoate) biosynthesis from triglyceride substrates',. Applied Microbiology and Biotechnology. 49(4), pp. 431–437.
7. *A.J*.F. Carvalho, in S. Ebnesajjad ,ed., Handbook of Biopolymers and Bio-degradable Plastics:Properties, Processing and Application, William Andrew Publishing,2012.
8. Adwitiya Pal, Ashwini prabhu, Avinash arun kumar, Badri rajagopal,Kajal dadhe, Vomsi ponnamma and Srividya shivakumar(2009) Optimization of Process Parameters for Maximum Poly(-β-)hydroxybutyrate (PHB) Production by Bacillus thuringiensis IAM 12077. *Polish Journal of Microbiology* . 58, (2),141- 149.
9. Aramvash Shahabi, Z., Dashti Aghjeh, S., Ghafari, M. D., (2015) 'Statistical physical and nutrient optimization of bioplastic polyhydroxybutyrate production by Cupriavidus necator', International Journal of Environmental Science and Technology. 12(7), pp. 2307– 2316.
10. (Bioplastic) and its Biodegradation by Pseudomonas Lemoignei and Aspergillus Niger ,Indian Institute of Crop Processing Technology ,Thanjavur ,Tamil nadu ,India.
11. Bernd H.A.Rehm, Polyester synthases:natural catalysts for plastics,Biuochem .J,376 (2003) 15-33.
12. Brandl,H., Grows, R . A.,Lenz , R .W .and fuller ,R . C.1990. Plastics from bacteria and for bacteria: polyhydroxyalkanoates as natural , biocompartable , bio degradable polyesters . Adv . biochem.Eng bio technol., 41:77-93.
13. Chaijamrus. S and N. Udpuay.(2008) "Production and Characterization of Polyhydroxybutyrate from Molasses and Corn Steep Liquor produced by *Bacillus megaterium* ATCC 6748". Journal of *Agricultural Engineering International: the CIGR E. Manuscript FP 07 030. Vol.*
14. *X*.Bioplastics market to Triple,2013,http;//www.european-biotechnology-news.com/news/news/2013-01/bioplastics –production-to-triple.html,accessed2015.
15 Castilho, L. R., Mitchell, D. A. and Freire, D. M. G. (2009) 'Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation', Bioresource Technology, 100(23), pp. 5996–6009..
16 Castro –sowynski S , Burdman S , Matan O , Okon Y (2009) natural functions of bacteria poly hydroxy Butyrate .Microbiolmonogr. doi : 10. 1007/978 -3-642-03287-5-3.
17 Chelala C., 2010. Rapid Urbanization has an Impact on Health. The Epoch times. www. Theepochtimes.com/n2/content/view/27602/.
18 Chen, J., Chen, G. (2001). 'Production of Poly-3-hydroxybutyrate by Bacillus sp' . JMa5 Cultivated in Molasses Media', 47(6), pp. 587–593
19 Das.Q J.U. chowdhury and M.N. Anwar.(2007). Isolation, Purification and Characterization of Biodegradable Polymer Producing Bacteria *Pseudomonas pseudomallei*. *International Journal of ChemTech Research*: 4 (1);430-437.
20 DiGregorio B.E.,2009. Biobased performance bioplastic: mirel.chemistry and biology. 16(1):1-2.
21 Eriksen, M., Lebreton, L.C.M., Carson, H.S., Thiel, M., Moore, C.J., Borerro, J.C., Galgani, F., Ryan, P.G., Reisser, J., (2014). 'Plastic Pollution in the World's', Microbiology Monographs. 14, pp 17-37.