Fuel

COUNTRY	SUBJECT AREA AND CATEGORY	PUBLISHER	H-INDEX
Netherlands Universities and research institutions in Netherlands Media Ranking in Netherlands	Chemical Engineering Chemical Engineering (miscellaneous) Chemistry Organic Chemistry Energy Energy Engineering and Power	Elsevier B.V.	256

Q1 Chemical Engineering (miscellaneous) best quartile

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Page: 1 of 1 (5 total assignments)

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Action Links	2	JFUE-D-23-01042R1	Research Paper	Hydrolysis of Camellia oleifera shell with crude enzymes from Aspergillus fumigatus for bioethanol production	May 30, 2023	Completed - Reject	May 24, 2023	May 24, 2023	Jun 01, 2023	May 30, 2023
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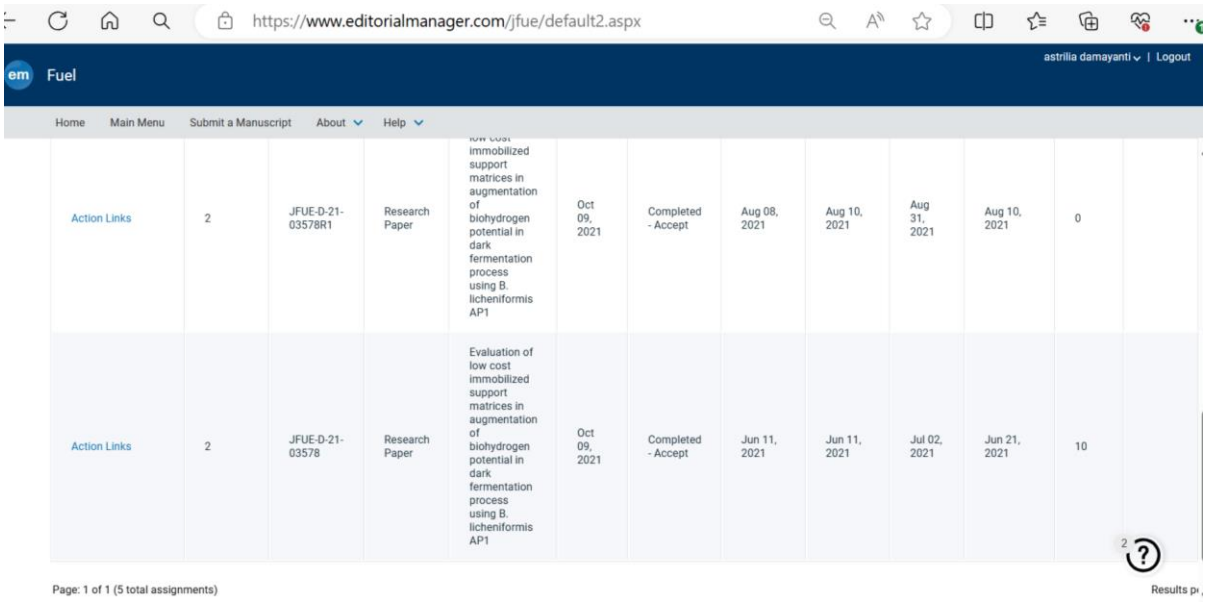
Action Links	2	JFUE-D-23-01042	Research Paper	Hydrolysis of Camellia oleifera shell with crude enzymes from Aspergillus fumigatus for bioethanol production	May 30, 2023	Completed - Reject	Mar 26, 2023	Mar 26, 2023	Apr 09, 2023	Apr 05, 2023
Action Links	2	JFUE-D-21-03578R2	Research Paper	Evaluation of low cost immobilized support matrices in augmentation of biohydrogen potential in dark fermentation process using B. licheniformis AP1	Oct 09, 2021	Completed - Accept	Sep 17, 2021	Sep 26, 2021	Oct 04, 2021	Sep 26, 2021



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"Evaluation of low cost immobilized support matrices in augmentation of biohydrogen potential in dark fermentation process using B. licheniformis AP1"

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	Original Submission
(Reviewer 1)	Major amendments required
astrilia damayanti (Reviewer 2)	Do not publish
(Reviewer 3)	Major amendments required
Author Decision Letter	Revise

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File Note

To Editor

writing the word "conclusion" in the discussion = page 12, 13, and 16

References

1. Said, Dzul Rashidi, Maswanna, and Salem did not used as a citation
2. Sagir et al (2018) is used as only 1 while in reference there are 2. which one is used in the script
3. Ghimire et al (2015) is written 2 times but doi are different
4. Jamali et al (2019) in the reference there are 2. which one is used in the script

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General

1. Actually this study did not need to be immobilized in cell traps because the research results of Sekoi et al (2018) page 14 shows that immobilization by adsorption was easier than cell immobilization.
2. there is no sequence of narration and argumentation
3. the phrase "previous study" is often used
4. The autoclave is working incorrectly. Not 40-50^{o}C but 121^{o}C for 15 minutes
5. This study does not explain the percentage distribution of nutrients, matrix, and cells in the fermentor
6. This study does not explained how long the fermentation process did and when to take samples
7. There are many typos
8. Often conclude in the discussion
9. It is inconsistent to use the words suspended cells/free cell/unimmobilized cells.
10. Inconsistent of citation
11. references should use a reference engine

Abstract

1. It should be explained how long it takes to fermentation
2. It should be explained the results of each matrix
3. It should be explained the pH of the fermentation product
4. Comparison of results should be with other matrices, not with controls

Introduction

1. There is no reason why choose foam as a place for cell growth
2. Matrix that is not related to this research from previous research should be ignored

3. The storyline is not coherent, for example from the matrix story and the addition of gold metal. There must be a reason for choosing the metal TiO_2-NP
4. There is no explain about the method used for cell traps
5. There is no explain about relation of VFA and pH on hydrogen production

Method

1. There is no information on the brand and the concentration of chemicals used, matrix acquisition, and the tools used to make cell immobilization
2. Page 5 section 2.3, how cells can grow during incubation while no food (substrate) is provided
3. Page 5 section 2.4 is not clear how it works and the need for the materials used
4. Page 5 section 2.4 ….autoclaved at 40-50°C ….Incorrect autoclave temperature. Means this work is also wrong.
5. Page 6 section 2.4 the concentration of $CaCl_2$ and TiO_2-NP has not been told in the introduction
6. Page 6 section 2.5. its work did not clear
7. Page 6 section 2.6.SEM testing is not explained on what day in each matrix

Result and discussion

3.1. Effect of immobilized matrices on biohydrogen potential

Page 7 Explanation of Figure 2 is not clear. High porosity was the best reason for the immobilization of bacteria in polyurethane foam. What does it mean??

Page 7 line 17 Suspended cells?? even though there is no explanation in method

The explanation of Fig.2 is not clear because of the inconsistent use of the word “suspended cell” in the description of Fig.2 (unimmobilized)

Page 7 line 23 Explanation Fig.1, has not shown the results and discussion as well as the results of H_2 with foam <<< coconut fiber while the error bar >>> coconut fiber

There are no explanations for Fig.3 and 4
Page 8 section 3.1 the narrative of previous study is not clear in its direction when it is associated with the results of this study.

3.2. Impact of immobilized matrices on end metabolites production, substrate degradation, and final pH

Page 9 row 7 there is no explanation as to why The highest substrate degradation (corn starch) was seen in foam support carrier coconut coir wood shaving control

Description Fig 1 … with inoculum volume 20% …should be … with inoculum 20% v/v …

Page 10 Fig. 2, 3, 4 There is no description of the magnification used in each image

Page 11 Table 1 The final pH for the foam matrix is the smallest while the hydrogen yield is "considered" large. Why?

3.3. Impact of immobilized alginate (B. licheniformis AP1 + TiO2-NP) on biohydrogen Production

The narrative in this sub-chapter does not emphasize the results and discussion of the obtained tests. Page 14 Fig.5 There are no results and discussions yet

3.4. Impact of immobilized alginate (B. licheniformis AP1 + TiO2-NP) on end metabolites production, substrate degradation and final pH

Table 3 cannot be used because the substrate, reactor type, immobilization matrix, and cells used are different from this study.

Fuel

Evaluation of low cost immobilized support matrices in augmentation of biohydrogen potential in dark fermentation process using B. licheniformis AP1

--Manuscript Draft--

Manuscript Number:	JFUE-D-21-03578
Article Type:	Research Paper
Keywords:	B. licheniformis AP1; Clean fuel production; Cell immobilization; Dark fermentation; Nanoparticle; Volatile fatty acids
Abstract:	In this study, immobilization techniques (cell adsorption and cell entrapment) for augmenting biohydrogen potential has been reported. The novelty of this research is to study the impact of waste matrices or carriers (coconut coir-CC, wood shaving-WS, foam-FM) and low cost carriers (alginate-AL as well as alginate immobilized TiO 2 -NP) for biohydrogen potential augmentation using B.licheniformis AP1. Alginate immobilized TiO 2 -NP varying in concentration (200 mg/L, 400 mg/L, 600 mg/L, 800 mg/L, and 1000 mg/L) was used due to its limited exposure in dark fermentation process and achieved a significant escalation in biohydrogen potential at 1000 mg/L TiO 2 -NP (2 mol/mol of glucose). The SEM observation displayed that foam was the best carrier for high cell adhesion on its surface in comparison to other carriers. Thus, the cell adsorption method is found highly effective as compared to the cell entrapment method for increasing biohydrogen potential. Maximum 2.07 mol/mol of glucose yield and a 1.6 fold enhancement were achieved in immobilized foam carrier in comparison to control. Majorly, acetic acid followed by butyric acid is analyzed as by-products at the end of dark fermentation.

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Highlights

Optimization of support matrixes was experimented for hydrogen augmentation.

Cell adsorption method is highly efficient in comparison to cell entrapment.

Foam matrix was the best matrix for higher cell density adsorption.

Highest biohydrogen production was reported as 109.5 mL/30 mL medium using faom carrier.

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Evaluation of low cost immobilized support matrices in augmentation of biohydrogen potential in dark fermentation process using *B. licheniformis* AP1

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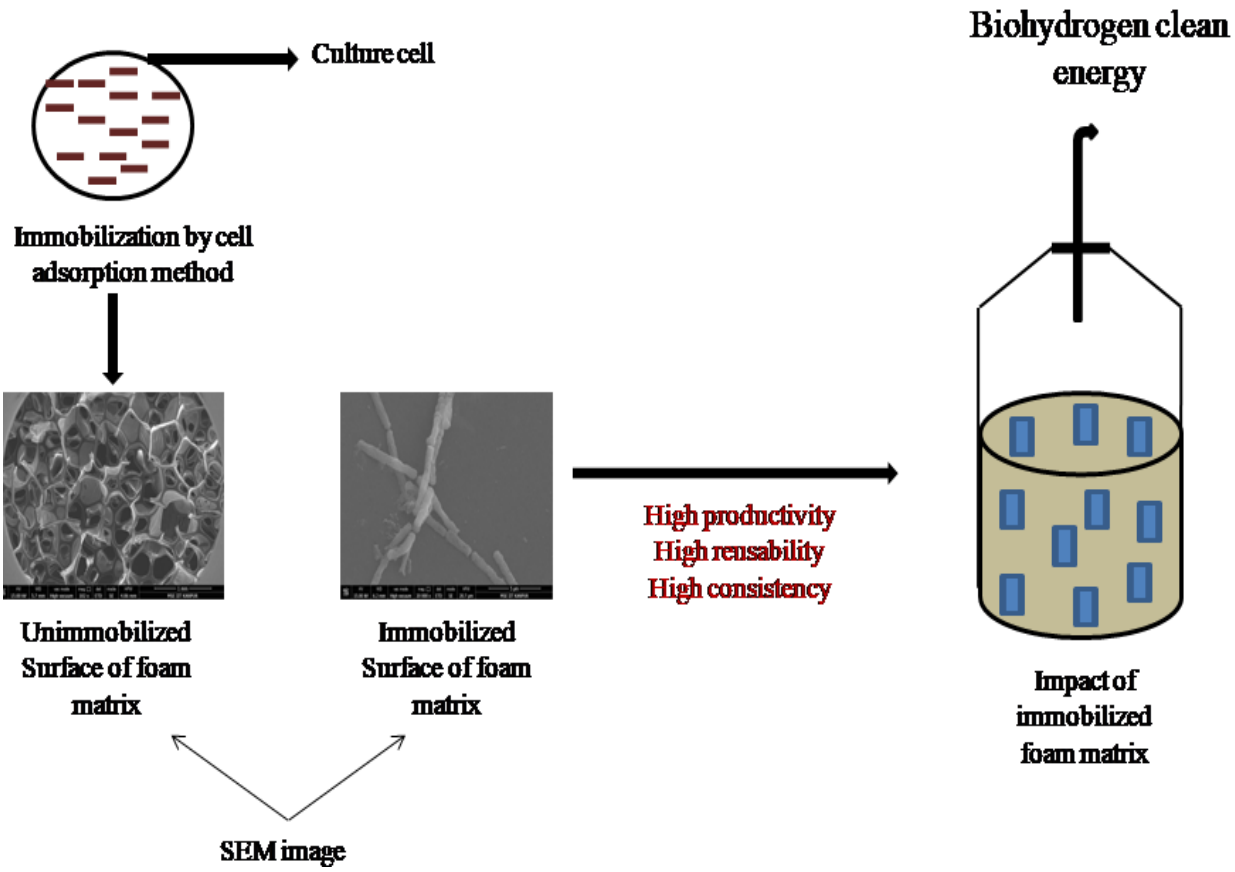
Abstract- In this study, immobilization techniques (cell adsorption and cell entrapment) for augmenting biohydrogen potential has been reported. The novelty of this research is to study the impact of waste matrices or carriers (coconut coir-CC, wood shaving-WS, foam-FM) and low cost carriers (alginate-AL as well as alginate immobilized TiO_2-NP) for biohydrogen potential augmentation using *B.licheniformis* AP1. Alginate immobilized TiO_2-NP varying in concentration (200 mg/L, 400 mg/L, 600 mg/L, 800 mg/L, and 1000 mg/L) was used due to its limited exposure in dark fermentation process and achieved a significant escalation in biohydrogen potential at 1000 mg/L TiO_2-NP (2 mol/mol of glucose). The SEM observation displayed that foam was the best carrier for high cell adhesion on its surface in comparison to other carriers. Thus, the cell adsorption method is found highly effective as compared to the cell entrapment method for increasing biohydrogen potential. Maximum 2.07 mol/mol of glucose yield and a 1.6 fold enhancement were achieved in immobilized foam carrier in comparison to control. Majorly, acetic acid followed by butyric acid is analyzed as by-products at the end of dark fermentation.

Graphical abstract



Keywords: *B. licheniformis* AP1; Clean fuel production; Cell immobilization; Dark fermentation; Nanoparticle; Volatile fatty acids

1. Introduction

The demand for renewable energy resources is being increased due to the rapid depletion of non-renewable resources and also due to increasing global warming, pollution, and acid rain (Rai et al., 2019, Thiyagarajan et al., 2019) etc. Hydrogen gas as a non-conventional energy source plays a vital role in current research aspects because of its high energy content (142 kJ/g), zero-emission of carbon (Rai et al., 2019) and with the help of fuel cell technology it is easily converted to electricity (Rambabu et al., 2020). There are various methods of hydrogen gas production like steam reforming, catalytic reforming, coal gasification, and water electrolysis (Zhang et al., 2020) but all these methods are responsible for greenhouse gas emission in the environment and also need high energy input. Researchers are targeting

on biological routes (instead of chemical route) like photo-fermentation (via photosynthetic bacteria) (Dai et al., 2021), dark- fermentation (via anaerobic bacteria) (Rangel et al., 2020) for hydrogen gas production because it is eco-friendly in nature and also sustainable for the environment. So, we are focusing here dark fermentation process for biohydrogen production owing to give high production yield (Margareta et al., 2020), and needs less input energy for the processing of fermentation. Dark fermentation mediated hydrogen production has remarkably reduced the bulk size of biowaste i.e. algae (Margareta et al., 2020), lignocellulosic waste (Jiang et al., 2019), food waste (Banu et al., 2020), and fruit waste (Mahato et al., 2020), and coffee Mucilage (Rangel et al., 2020) to valuable products like hydrogen gas, acetic acid, butyric acid, etc. Now, the major challenge for researchers is to achieve high hydrogen yield near to theoretical yield using bio-wastes as substrate (Mahato et al., 2020, Rangel et al., 2020, and Margareta et al., 2020), waste matrices (Gokfiliz et al., 2017, Patel et al., 2020), and nanoparticles through different biological routes (Pugazhendhi et al., 2019, Vamvasakis et al., 2016). Various organic and synthetic matrices such as lignocellulosic biomass, foam, thin slices of wood chip, polythene, paper, synthetic or cotton cloth are thrown in the environment after first to many time use. These waste matrices when left in the environment without any precaution, it cloges various rivers, ponds, and sewage water flow and causes serious hindrance in flow of natural environment process. So, our responsibility is to use such waste matrices for the utilization of useful product generation. Hence, immobilization techniques are being processed for utilization of these matrices for increasing the high yield output. To retain high biomass concentration, researchers are looking towards immobilization techniques (like entrapment, adsorption, encapsulation, and containment within synthetic polymer) (Zhang et Al., 2017) for improving H_2 yield by hydrogen-producing strain or modified strain (via metabolic engineering technique). Immobilization techniques are successful in improving biohydrogen yield to some extent due

to short lag phase, high substrate conversion efficiency, reusability, and less microbial contamination (Sekoai et al., 2018, Kourkoutas et al., 2004). Granular activated carbon was proved as an excellent support carrier for microbial growth (Jamali et al., 2016). Immobilization of phototrophic bacteria using polyvinyl alcohol (PVA) cryogel as a support material showed good results in biohydrogen production (Du Toit et al., 2020). Various support carriers like alginate (Zhang et al., 2017), agar (Sagir et al., 2018), activated carbon (Jamali et al., 2019), expanded clay (De Amorim et al., 2009), and cellulose (Srivastava et al., 2019), are reported for immobilization of microbes. Metal ions also play a vital role in improving high yield when incorporated within alginate beads (Seelert et al., 2015). Since one decade, nanoparticles are on demand even at low concentration due to its surface effect and quantum size effect and finally leading to improvement in the kinetics of microbes via electrons transfer to the acceptor in dark fermentation. A previous study has proved that the addition of 5nm gold particle directed the pathway towards VFA production and achieved H_2 yield 4.48 mol/mol of sucrose (Zhang et al., 2007) while inclusion of 20 nmol L^{-1} concentration silver nanoparticle obtained 2.48 mol H_2/mol glucose (Zhao et al., 2013). One of the previous studies has reported that chitosan coated Fe_3O_4@SiO_2 NPs has shown a great efficiency in biohydrogen production (Shanmugam et al., 2020).

The current research aims to study the impact of various waste support carrier and low cost support for biohydrogen potential augmentation. Exploration of TiO_2-NP incorporated in sodium alginate was also evaluated for the enhancement of biohydrogen potential. The comprehensive impact of immobilized TiO_2-NP was first time addressed in dark fermentation process using *B. licheniformis* AP1.

2. Methodology

2.1. Microorganism and culture media composition

Bacillus licheniformis AP1 (Srivastava et al., 2017) was used for dark fermentation as seed culture. 20% v/v of 18 hr grown culture was taken as inoculum for all the batch experiments. 1% w/v corn starch was used as an energy source and supplemented with **macronutrients:** NH_4Cl (8.1 g/L), KH_2PO_4 (9.4 g/L), K_2HPO_4 (9.4 g/L), NaCl (0.4 g/L), $CaCl_2$ $2H_2O$ (0.5 g/L), and $MgCl_2.6H_2O$ (0.93 g/L) **micronutrients:** $FeSO_4.2H_2O$ (50 μg/L), $NiCl_2.6H_2O$ (350 μg/L), $NaMoO_4$ (90 μg/L), $CoCl_2.4H_2O$ (200 μg/L), $MnCl_2.4H_2O$ (300 μg/L), **multivitamin:** (becosule capsule) (510.0 μg/L).

2.2. Selection and preparation of matrices for immobilization

Three matrices i.e. foam, coconut coir, and wood-shaving were selected based on easy availability of waste material**.** Each piece of foam, coconut coir, and wood-shaving was cut into square pieces (size-0.5 cm * 0.5 cm * 0.5 cm) and was used as a support carrier for the immobilization of *B.licheniformis* AP1. All the pieces of coconut coir and wood-shaving should be autoclaved before using as a support carrier for immobilization and foam pieces were dipped into absolute alcohol for contamination removal. Sodium alginate supplemented with TiO_2-NP was also used for immobilization.

2.3. Immobilization of *B. licheniformis* AP1 by cell adsorption method

10 pieces of each waste support carrier were put in 60 mL serum bottle with a working volume of 30 mL fermentation medium and was inoculated with 20% v/v *B.licheniformis* AP1 and keep it for 18 hours in incubator for the growth of microbial film over the matrices. Now, take out all the pieces of foam, coconut coir, and wood-shaving from the serum bottle and set it (immobilized carriers or matrices) for the bio-hydrogen production with fresh 30 ml fermentation medium.

2.4. Immobilization of (*B. licheniformis* AP1+ TiO_2 –NP) by entrapment method

Hydrogen producing *B. licheniformis* AP1 (20% v/v) and TiO_2-NP [39] were quickly mixed with autoclaved sodium alginate solution at 40-50 °C to make the different final

concentration of TiO_2-NP (200 mg/L, 400 mg/L, 600 mg/L, 800 mg/L, and 1000 mg/L). The solution mixture was dropped into chilled 0.5M $CaCl_2$ with the help of a syringe to form beads (2-3 mm in diameter). Now immobilized beads of varying concentration of TiO_2-NP were put into different 60 mL serum bottle with working volume of 30 mL fermentation medium for the experimental (bio-hydrogen production) set up.

2.5. Experimental set up

60 mL serum glass bottles stoppered with rubber caps were used as set up for all the experiments. The batch serum bottle has 30 mL working volume with 30 mL headspace. To make the environment anaerobic, N_2 gas was used for removing all the dissolved gas with sparging time of 1 min. 12 mL leakage - proof disposable syringes were used for the collection and measurement of gas by plunger displacement method (Rai et al., 2019). All the experiments were studied in duplication (replication) for statistical analysis and results are reported as average of duplicates ± standard deviation. The standard deviation of mean values was calculated using Microsoft Excel version using Microsoft Excel 2007.

2.6. Analytical methods

The accumulated gas was analyzed by using GC (Agilent 7890, USA) equipped with a thermal conductivity detector with the help of nitrogen as a carrier gas at a flow rate of 25 mL/min. The temperatures for injector, oven, and detector were set as 80°C, 90°C, and 110°C respectively. Volatile fatty acid (VFA) was detected by using GC equipped with a flame ionized detector and nitrogen was used as a mobile phase at the flow rate of 1.5 mL/min. The operating temperatures for the oven, detector, and injector were set as 110°C, 250°C, and 250°C respectively. Liquid samples were taken after the fermentation process end for the VFA analysis and sugar analysis. The liquid sample was centrifuged at 8000 rpm for 15 min. The clear supernatant was taken for VFA and sugar estimation. Reducing sugar was estimated by 3, 5-dinitrosalicylic acid (DNS) method (Deshavath et al., 2020) using a Cary

60 UV–Vis spectrophotometer (Agilent USA). The initial and final pH of the medium was measured using Eutech (Merck) pH meter.

2.7 Culture cell observation

Cell immobilized and unimmobilized matrices were observed by using a scanning electron microscope (SEM) (Stereosan 420; Leica Cambridge Instruments). All the immobilized and unimmobilized matrices were gently cleaned with phosphate buffer solution. Gluteraldehyde (3% v/v) (Penniston et al., 2018) was used for fixing the samples (immobilized and unimmobilized matrices) and left it for 4 to 5 hr. The fixed samples were serially dehydrated by 10%, 20%, 40%, 60%, 80% and 100% ethanol (Zhang et al., 2007) and then dried in oven (NSW - 143 Oven Universal, Super Deluxe, India) at 50 to 55 °C for 24 hr.

3. Results and discussion

3.1. Effect of immobilized matrices on biohydrogen potential

Three support matrices i.e. polyurethane foam, coconut coir, and wood-shaving were used for immobilization of *Bacillus licheniformis* AP 1 on its surface. SEM images of all the matrices (polyurethane foam, coconut coir, and wood-shaving) with and without immobilization were shown in Fig. 2, Fig. 3, and Fig. 4. Biohydrogen gas production was analyzed using these immobilized matrices in comparison to the suspended cell. Fig. 2 clearly shows that polyurethane foam is the best carrier for the retention of *Bacillus licheniformis* AP 1. High porosity was the best reason for the immobilization of bacteria in polyurethane foam. A large no of pores in the foam support carrier created a high surface area that accumulated large no of bacteria by the adsorption process. Thus, high density of immobilized *Bacillus licheniformis* AP 1 on foam matrice utilized substrate (corn starch) in a better way in comparison to other matrices for enhancing hydrogen production. The results (Fig.1) have shown that the types of support matrices affected the productivity of gas due to surface characteristics such as surface roughness, and surface area. Cappelletti et al. (2012) reported

that biomax (highest surface area) was the best carrier among four different carriers (Siporax, siporax cylinders, Glaxstone, biomax) for the immobilazion of *Thermotoga neapolitana* for augmenting the hydrogen production efficiency through the dark fermentation process. As concern about the hydrogen specific rate, biomax carrier generated highest hydrogen specific rate (22 μmol_{H2} mg^{-1} protein h^{-1}). A previous study reported (Muri et al., 2018) that Mutag BioChip™ support material exhibited effective performance in adsorption of microbes (hydrogen evolving) on its surface owing to various numerous pores and achieved maximum yield (1.80 mol H_2/mol glucose). While other two support materials such as expanded clay (1.74 mol H_2/mol glucose) and activated carbon (1.46 mol H_2/mol glucose) were not so efficient as Mutag BioChip™ in terms of retention of hydrogen-producing microbe (Muri et al., 2018). In another study, three different carriers (Polyester fiber, activated carbon and corn stalk) were used for immobilization and concluded from the SEM image that corn stalk was the best carrier in comparison to polyester fiber, and activated carbon owing to have numerous pores which provide extra surface area for the attachment of *Bacillus cereus* A1 and *Brevumdimonas naejangsanensis* B1 (Ma et al., 2017). A study reported that hydrogen production efficiency was increased when expanded clay was used as a support carrier in comparison to polystyrene material in anaerobic fluidized bed reactor (AFBR). The surface roughness and creviced surface of the support carrier was the best reason for the attachment of microbes to the expanded clay than the polystyrene because creviced surface protects the immobilized bacteria from the shear force (Barros et al., 2010). One previous study proved that higher productivity in hydrogen production is associated with immobilized cells over suspended cells (Ma et al., 2017).

3.2. Impact of immobilized matrices on end metabolites production, substrate degradation, and final pH

Acetate (prominent metabolite) and butyrate (second most metabolite) were observed at the end of fermentation processon, suggesting acetate/butyrate type of fermentation process. An acetate/butyrate type of fermentation was analyzed in all the tested treatment including free cell as a control. The minimum and maximum acetate/butyrate ratio were observed in control and immobilized polyurethane foam (support carrier). Production of acetate and butyrate in the spent media favors the generation of hydrogen gas. The highest substrate degradation (corn starch) was seen in foam support carrier > coconut coir > wood shaving > control (free

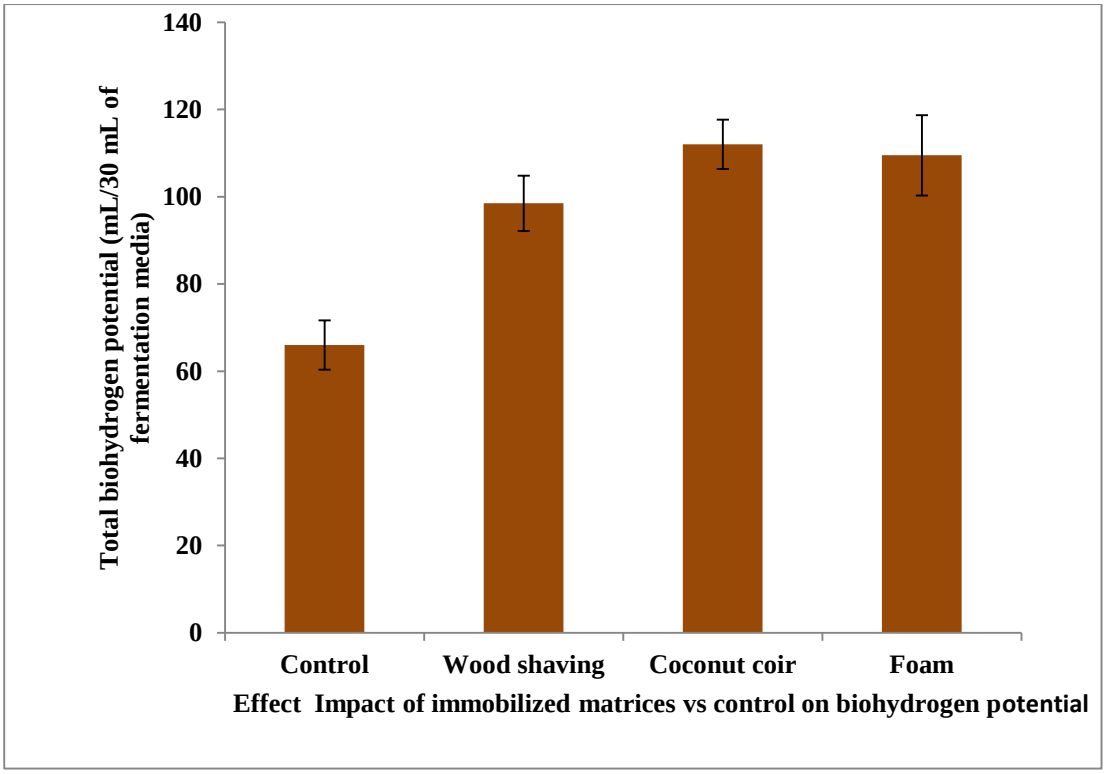


Fig. 1. Effect of immobilized matrices on biohydrogen potential using corn starch substrate with inoculum volume 20%, inoculums age 18 hr, temperature 38±2 °C, and pH 6.5

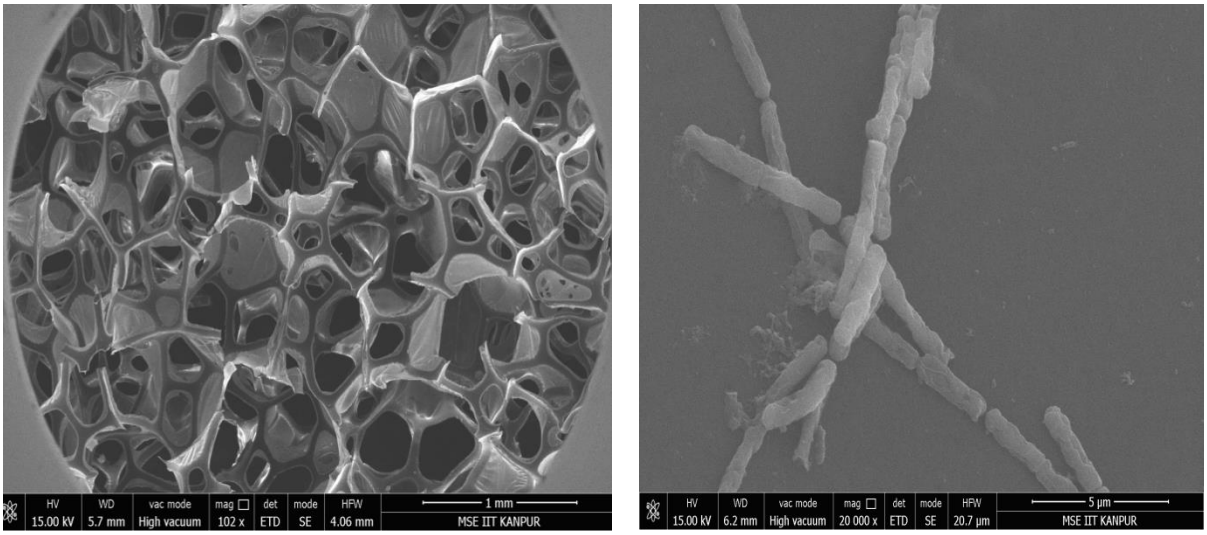


Fig. 2. SEM image of unimmobilized and immobilized foam

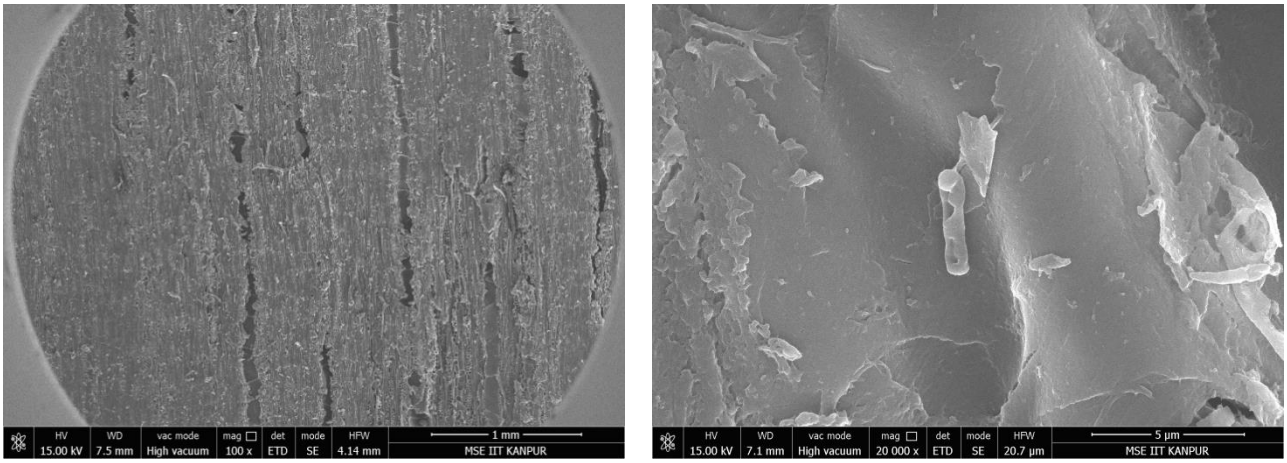


Fig. 3. SEM image of unimmobilized and immobilized wood shaving

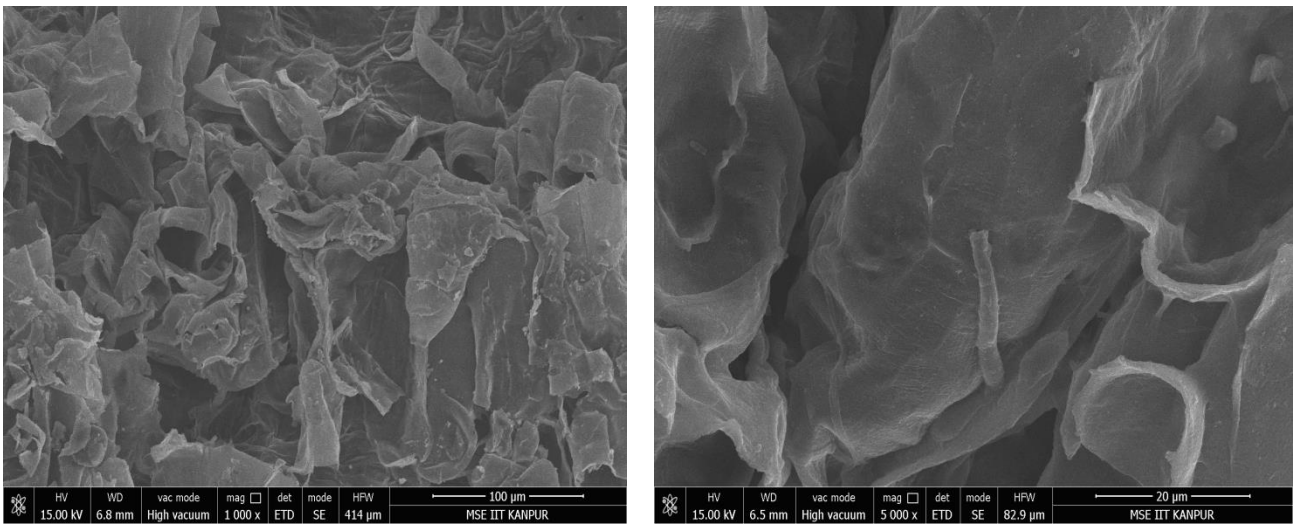


Fig. 4. SEM image of unimmobilized and immobilized coconut coir

cell) as shown in Table 1. In addition, maximum retention of *Bacillus licheniformis* AP 1 on foam carrier is proved in Fig. 2, and plausible reason for this retention is due to the surface roughness of the carrier. So, the bacterium due to its maximum in numbers majorly contacts with the substrate and mediates hydrogen evolution by maximal degradation of substrate and simultaneously another by-products such as VFAs (acetic acid, butyric acid, and isobutylic acid) are also found in maximum concentration that lowers the pH of the fermentation medium (4.42 pH) in case of foam support carrier. A research reported that VFAs produced during fermentation process attribute to decrease in pH (Sekoai et al., 2016) of the fermentation broth that inhibits the growth and metabolism of bacteria and finally cease the production of biohydrogen gas.

The overall concentration of VFAs was found highest when the foam was used as a support carrier, probably due to large substrate degradation that is performed by densely immobilized bacteria adsorbed to the foam support carrier.

Table 1 Distribution of VFAs associated with various matrices

Type	Acetic acid mg/L	Butyric acid mg/L	Isobutylic acid mg/L	Substrate degradation (%)	Initial pH	Final pH
Control	2931±138	1924±119	691±57	64%	6.5	6.21±0.21
Woodshaving	3753±101	2042±134	587.4±60	71%	6.5	5.57±0.24
Coconut coir	4082±133	2164.6±256	1046±107	75.4%	6.5	5.04±0.19
Foam	5102±162	2002±158	1549±129	90.66%	6.5	4.42±0.14

3.3. Impact of alginate immobilized (*B.licheniformis* AP1 + TiO_2-NP) on biohydrogen production

The entrapment of biohydrogen producing culture cells along with the different concentrations of TiO_2 nanoparticle in alginate has good impact on total biohydrogen

potential. For an efficient fermentation process, the support material should contain good permeability that helps in the transfer of nutrients and substrate from fermentation medium to immobilized cells for regulating the metabolic process. A study suggests that the cell entrapment through effective support material such as alginate, cellulose, agar, polyvinyl, carrageenan, polypropylene, and polyacrylamide make a protective barrier around the cell to improve its viability and longevity (Martins et al., 2013). Among them, alginate is highly effective for entrapment owing to be cheap in price as well as also effective for cell function inside the alginate beads in the dark fermentation process, and alginate immobilized cells are reusable. Polydimethylsiloxane was utilized as a support carrier for culture cell immobilization in augmenting biohydrogen production rate from palm oil effluent waste (Ismail et al., 2011). One previous study reported two-fold augmentation in biohydrogen production when activated carbon immobilized in calcium alginate beads (Wu et al., 2002). Exploration of metal ions use in alginate beads for high yield (2.1 mol H_2/mol glucose) achievement is also analyzed (Seelert et al., 2015). One study explored the use of polyethylene glycol immobilized *Clostridium* LS2 cells for maximizing biohydrogen production (Singh et al., 2013). Titanium oxide and chitosan immobilized in alginate beads enhanced three-fold biohydrogen production (Wu et al., 2006). While supporting the previous study (Wu et al.,2006), the result of our present study showed that the different concentrations of titanium oxide entrapped in alginate beads also enhanced biohydrogen production in comparison to control. At a lower concentration of TiO_2 (100 mg/L), the increase in biohydrogen gas potential was significantly seen as compared to the control test. So, it concludes that the enzymatic enhancement (Formate Hyrogen Lyase, and Pyruvate Formate Lyase) (Shanmugam et al., 2020, Kumar et al., 2019) due to nanosized NPs (quantum size effect and high surface effect) (Zhang et al., 2007) could be the plausible reason of TiO_2-NP addition on biohydrogen enhancement. So, it confirms the positive effect

of TiO_2-NP on biohydrogen production. It has been analyzed from Fig. 5 that biohydrogen potential increases as the concentration of titanium oxide increases. However, biohydrogen potential was observed low at 100 mg/L TiO_2-NP due to poor synergy between *B.licheniformis* AP1 and TiO_2-NP at this minimum concentration (100 mg/L).

Additionally, the highest biohydrogen potential was observed at highest concentration of titanium oxide (i.e. 1000 mg/L) due to positive interaction between *B.licheniformis* AP1 and TiO_2-NP by improving electron transfer efficiency.

As compared to other nanopaticles i.e. iron, nickel, and gold (Kumar et al., 2019) TiO_2-NP has limited impact on biohydrogen evolution in current research owing to bactericidal activity (Li et al., 2011), lower electrical and thermal conductivities (Hsieh et al., 2016).

However, the highest yield in the form of total biohydrogen potential was seen in the adsorption method in comparison to the cell entrapment method in this study. So, the SEM image of the beads supplemented with *B.licheniformis* AP1 + TiO_2 –NP has been ignored. Since the process of gas formation from the *B.licheniformis* AP1 + TiO_2 immobilized beads was started after 12 hr of the experiment set up. But some of the scientific phenomena were observed during the whole process of fermentation such as few beads were started to move upward after a few hours of fermentation process started. While some beads were found near the fermentation media's surface and another few beads were observed distorted at the end of the fermentation reaction. So, it is concluded that the beads observed near the fermentation media's surface were not able to release gas efficiently due to low particle density (Wu et al., 2002). In addition, distortion of the beads' structure was due to aggregation of gaseous product inside the beads that increases the inner pressure of the beads and causes splitting, and damages to immobilized beads (Wu et al., 2002).

In cell entrapment, various factors (pore size, bead size, types of substrate for immobilization, synthesis method, and stability) play a key role in improving biohydrogen production yield.

Sometimes, alginate beads undergo various limitations such as poor porosity, as well as low mechanical stability that lower down the production yield (Sekoai et al., 2016). Hence, one of the major reasons behind good productivity observed in adsorption method was due to direct contact of *B.licheniformis* AP1 to the fermentation media that can be easily metabolized by the bacterium. The immobilization of culture cells is easy in the adsorption method in comparison to cell entrapment (Sekoai et al. 2018).

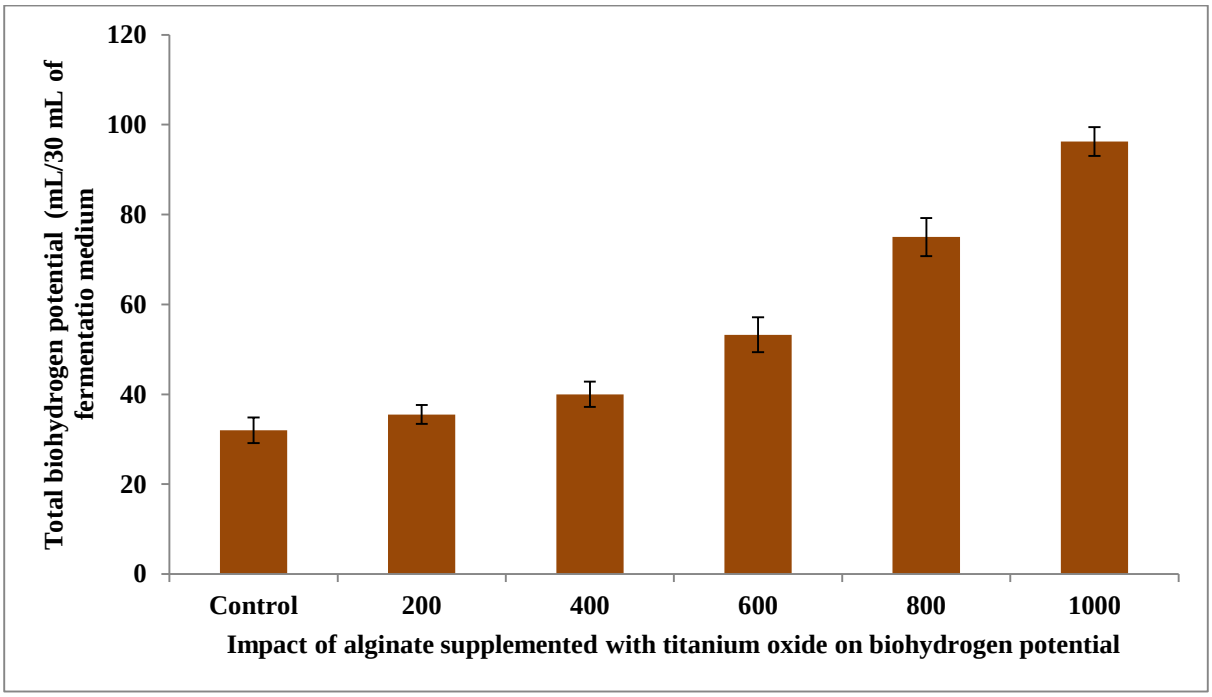


Fig. 5. Effect of various TiO_2 –NP (mg/L) on biohydrogen potential using corn starch substrate with inoculum volume 20%, inoculums age 18 hr, temperature 38±2 °C, and pH 6.5

3.4. Impact of alginate immobilized (*B. licheniformis* AP1 + TiO_2-NP) on end metabolites production, substrate degradation and final pH

Nanoparticle use has proved a tremendous effect in improving biohydrogen production in comparison to free cells in the various previous study. The involvement of nanoparticles in the fermentation process have confirmed that it actively participates in improving the bioactivity of bacteria by transferring the electron (Gadhe et al., 2015). In addition, a study

regarding alginate entrapped titanium oxide nanoparticle is limited in the dark fermentation process. Hence, alginate supplemented with different concentrations of titanium oxide nanoparticle (TiO_2-NP) was studied in this research and analyzed that alginate beads immobilized with titanium oxide were effective in comparison to alginate beads without titanium oxide nanoparticle (TiO_2-NP). The impact of different concentrations of titanium oxide nanoparticles on end-product distribution is shown in Table 2.

Table 2 Distribution of VFAs associated with various concentration of titanium oxide vs control

Type	Acetic acid mg/L	Butyric acid mg/L	Isobutylic acid mg/L	Substrate degradation (%)	Initial pH	Final pH
Control without TiO_2 NP	134±13.4	0.00	0.00	41.55%	6.5	6.34±0.27
TiO_2 200 mg/L	185.3±22.1	0.00	0.00	43.6%	6.5	6.22±0.16
TiO_2 400 mg/L	217±42.4	0.00	0.00	46.23%	6.5	6.17±0.22
TiO_2 600 mg/L	442.4±90	0.00	435.88±48.7	50.2%	6.5	6.01±0.18
TiO_2 800 mg/L	342±40.3	0.00	239.7±37.3	56.02%	6.5	5.83±029
TiO_2 1000 mg/L	613.69±49.3	561.5±95.8	21467±355.67	63.3%	6.5	5.62±0.23

It is clear from Table 2 that immobilized nanoparticle has a potential effect on end-product distribution, substrate degradation, and final pH as compared to control. As the concentration of titanium oxide increases from 200 mg/L to 800 mg/L the distribution of acetic acid end product increased. The butyric acid (561.5mg/L butyric acid) was observed only at the highest concentration (800 mg/L) of titanium oxide and found negligible from 200 mg/L to 600 mg/L titanium oxide. Acetate/butyrate type of fermentation process was found as the concentration of titanium oxide increased from 800 mg/L to1000 mg/L. It is reported that the production of acetate and butyrate efficiently supports biological biohydrogen production

(Ghimire et al., 2015) because both the metabolites are metabolically favourable for dark fermentative hydrogen production (Lee et al., 2006). It concludes that the highest yield in the form of biohydrogen potential was achieved at 1000 mg/L titanium oxide. Camparision of various matrices with corresponding yield is shown in Table 3.

Table 3 Comparative study of different support matrices and microbes used for biohydrogen production

Immobilized cells	Support material	Reactor type	Hydrogen yield	Carbon source	References
Sludge	Granulated activated carbon	Fluidized bed reactor	1.24 mol hydrogen/mol sugar	Palm oil mill effluent	Jamali et al.,2019
Enterobacter aerogenes	Multi-walled carbon nanotube (MWCNT-COOH)	Batch	2.2 mol/mol glucose	Glucose	Boshagh et al., 2019
Enterobacter aerogenes	Treated carbon fiber	Batch	2.56 mol/mol glucose	Glucose	Boshagh et al., 2019
Bacillus cereus strain A1 and *Brevundimonas naejangsanensis* strain B1	Corn stalk	Batch	1.81 mol H_2/mol glucose	Corn starch	Wang et al., 2018
C. acetobutylicum strain CICC 8012	Brick	Batch	1.81 mol H_2/mol glucose	Glucose	Liu et al., 2019
Sludge	GAC–Alg beads (Granulated activated carbon-Alginate)	Batch	2.09 ± 0.22 mol H_2/mol sugar	Glucose, Xylose	Dzul et al., 2020
Anaerobic granules	Polyethylene	Fixed bed reactor	0.83 mol H_2/mol glucose	Glucose	Kumar et al., 2017
Awamori and A. oryzae	Activated carbon	continuous mixed immobilized sludge reactor (CMISR)	85.6 mL/g food waste	Food waste	Han et al., 2015
Clostridium sp. LS2	Polyethylene glycol	Continuous reactor	0.31 L H_2/g chemical oxygen demand	Palm oil mill effluent (POME)	Singh et al., 2013
Anaerobic sludge	Mutag BioChip, expanded clay	Anaerobic packed-	1.80 mol H_2/mol glucose	Glucose	Muri et al., 2018

	and activated carbon	bed reactor			
B. *licheniformis* AP1	Foam , Coconut coir, Wood shaving, and alginate supplemented with TiO_2	Batch reactor	109.5 mL H_2/30 mL of fermentation medium by foam support carrier or 2.07 mol hydrogen/mol of glucose by using foam support carrier and 96.25 mL H_2/30 mL of fermentation medium at 1000 mg/L TiO_2-NP or 2 mol/mol of glucose at 1000 mg/L TiO_2-NP	Corn starch	- Present study

4. C**onclusion**

Intensifing biohydrogen production through fermentation process (biological method) is a major task and application of immobilization techniques in hydrogen production system is burgeoning area for escalating biohydrogen potential. However, enhancing biohydrogen potential in immobilized system is challanging task owing to various factors such as pH, temperature, characteristics of support matrices, substrate variety and its concentration. In this study, waste support matrices (foam, coconut coir, and wood-shaving), and low-cost support carrier (alginate) were used. The research outcome exhibited that the characteristics of the matrices play a significant role in augmentation of biohydrogen potential and foam was the best support carrier for the immobilization of *B. licheniformis* AP1 and also for maximizing biohydrogen potential as compared to other matrices. The highest hydrogen potential obtained with foam support carrier was 109.5 mL/30 mL of fermentation medium.

Batch fermentation process with control (suspended cells) displayed hydrogen potential (66±5 mL/30 mL of fermentation medium), which is relatively very low in comparision to results that is obtained from foam support carrier. In concise, foam can be used as a potential support carrier in fermentation process for large scale biohydrogen potential.

Credit author contribution statement

Priya Rai: Investigation, Creation of model, Experimental work, Analysis, Validation, Data curation, Writing - original draft, and Editing. **Ashutosh Pandey:** Review. **Anjana Pandey:** Supervision**,** and Review.

Declaration of competing Interest

The authors declare that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

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